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Award Number: W81XWH-04-1-0757

TITLE: Characterization of Fatty Acid Synthase (FAS) as a Predictive Marker of Breast Cancer Sensitivity to Chemotherapy

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REPORT DATE: November 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

x Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. Agency Use Only (Leave blank)	2. Report Date	3. Report Type and Period Covered (i.e., annual1 Jun 00-31 May 01)
	November 2005	Final 1 September 04-31 August 05

4. Title and Subtitle
Characterization of Fatty Acid Synthase (FAS) As a Predictive Marker of Breast Cancer Sensitivity to Chemotherapy

5. Award Number
W81XWH-04-1-0757

6. Author(s)
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8. Performing Organization Report Number (Leave Blank)

9. Sponsoring/Monitoring Agency Name and Address
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. Sponsoring/Monitoring Agency Report Number

11. Supplementary Notes (i.e., report contains color photos, report contains appendix in non-print form, etc.)
None

12a. Distribution/Availability Statement
Approved for public release; distribution unlimited

12b. Distribution Code

13. SUPPLEMENTARY NOTES

14. Abstract

The expression and activity of Fatty Acid Synthase (FASN), the sole protein in the human genome capable of *de novo* FA biogenesis, is extremely low in nearly all non-malignant adult tissues, whereas significant overexpression and concurrent increased activity of FASN represents an early and nearly universal phenotypic alteration in most human cancers. This creates a large therapeutic index and, therefore, FASN-driven endogenous FA synthesis pathway may provide a number of avenues of future exploration applicable to the prevention andjor treatment of cancer disease. Upon FASN blockade, cancer cells stop proliferating and ultimately die mainly through the process of apoptosis. Since solid tumors display a complex drug resistant phenotype that involves inherent an acquired mechanisms, and because up-regulation of FASN associates with poorer prognosis in important subsets of human carcinomas, we formerly theorized that tumor-associated FASN plays a causal role in the response to anti-cancer treatments. In the last few years our group has evaluated whether chemical FASN blockers as well as RNA silencing interference techniques directed against *FASN* gene may provide a mean to increase efficacy Qver existing therapy in human breast carcinomas. Her, we perimentally review how the FASN-related ''lipogenic phenotype" actively regulates the efficacy of anthracyclines (i.e., doxorubicin), microtubule-interfering agents (i.e., paclitaxel, docetaxel, and vinorelbine), nucleotide analogues (i.e., 5-Fluorouracil), and monoclonal antibodies (i.e., trastuzumab) in cultured breast cancer cells. Inherent and acquired drug resistance hampers successful treatment in many human malignancies, and its prevention or reversal is still awaiting new therapeutic strategies or pharmaceuticals. While current research is focused on producing the ideal FASN blocker and it is hoped that the latest 4,5 A resolution X-ray crystallographic map of mammalian FASN will lay a basis for efforts at structure-based drug design with this target, we present evidence to suggest that FASN-catalyzed endogenous FA biogenesis is a previously unrecognized respondent mechanism to drug-induced cell injuries. The fact that breast cancer cells differing in FASN levels significantly differs as well in the sensitivity of breast cancer cells to chemo-, endocrine- and biological response modulators should impose new challenges for the future use of anti-FASN strategies in the chemoprevention or curative treatment of breast cancer disease.

15. Subject Terms: Fatty acid Synthase; Metabolism; Chemotherapy; Breast Cancer

18. Number of Pages
36

16. SECURITY CLASSIFICATION OF:

19a. NAME OF RESPONSIBLE PERSON : USAMRMC

a. Security Classification of Report	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF	19b. TELEPHONE NUMBER
Unclassified	Unclassified	Unclassified	UU	

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INTRODUCTION

One perception of tumour cell metabolism ascertains that, while an altered metabolism is not the cause of malignancy, without the required metabolic transformation, the neoplastic cell cannot successfully elicit its malignant capabilities. This assumption, commonly applied to the shift toward lactate production in cancers ("glycolytic switch") even in the presence of adequate oxygen (*i.e.*, the "Warburg effect"), appears to be also true for FASN-catalyzed *de novo* FA biogenesis ("lipogenic switch"), another metabolic requirement of malignant cells. Thus, it is usually accepted that the same disturbances in signaling pathways responsible for oncogenic transformation also contribute to the increased FASN-driven lipogenesis in tumour cells. Alternatively, it has been proposed that the "glycolytic phenotype" is not a secondary phenomenon that results from induction of some other pathway during carcinogenesis. Rather, it is directly selected because it provides a growth advantage and its acquisition might be achieved through multiple mechanisms. Recent clinical and basic research studies have also evidenced that up-regulation of FASN gene expression and FASN biosynthetic activity are molecular events accompanying the pathogenesis and natural history of cancer disease. Therefore, the "lipogenic phenotype" in tumour cells appears to play advantageous functions unrelated to its well recognized anabolic-energy-storage role in normal cells.

Thought specific FASN blockade efficiently revert oncogene-driven transformed phenotypes such as enhanced cell proliferation, increased cell survival and anchorage-independent growth, FASN-catalyzed endogenous FA biogenesis begins to be considered, similarly to aerobic glycolysis, a metabolic hallmark necessary in the maintenance and enhancement of the malignant transformation state. Moreover, the consequences of FASN inhibition differ widely between cancer and normal cells. Thus, FASN inhibition rapidly induces apoptosis in cancer cells both *in vitro* and *in vivo*, with no significant effects toward normal cells, thus implying the reliance of tumor proliferation and survival on FASN-dependent *de novo* FA synthesis. Indeed, tumor-associated FASN (oncogenic antigen-519) canonically functions as an anti-apoptotic protein. Given that FASN expression is high in a biologically aggressive sub-set of human carcinomas, including breast cancer, together with the clinical observation that breast cancer patients with a higher level of FASN have poorer prognosis compared with the ones with lower levels, we and other groups envisioned that FASN may likely play an important role in drug resistance. This research project has experimentally assessed how chemical FASN blockers as well as RNA silencing interference techniques directed against *FASN* gene actually provide a mean to increase efficacy over existing therapy in human breast carcinomas.

BODY

Fatty Acid Synthase (FASN): A novel target for drug development

De novo synthesis of fatty acids in the cytosol of animal cells is carried out by the 250- to 270-kD multifunctional, homodimeric Fatty Acid Synthase (FASN). FASN consists of two identical multifunctional polypeptides, in which three catalytic domains in the N-terminal section (β-ketoacyl synthase (KS), malonyl/acetyltransferase (MAT), and dehydrase (DH)), are separated by a core region of ~600 residues from four C-terminal domains (enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP) and thioesterase (TE); ["Fig. (**1a,b**)]". FASN synthesizes long-chain fatty acids, mainly palmitate, *de novo* from the substrates acetyl-CoA, malonyl-CoA, and NADPH through its six active sites, *i.e.*, MAT, KS, KR, DH, ER and TE:

$$CH_3COS\text{-}CoA + 7\ HOOCCH_2COS\text{-}CoA + 14\ NADPH + 14\ H^+ \rightarrow$$
$$CH_3CH_2(CH_2CH_2)_6CH_2COOH + 14\ NADP^+ + 6\ H_2O + 8\ CoA\text{-}SH + 7\ CO_2$$

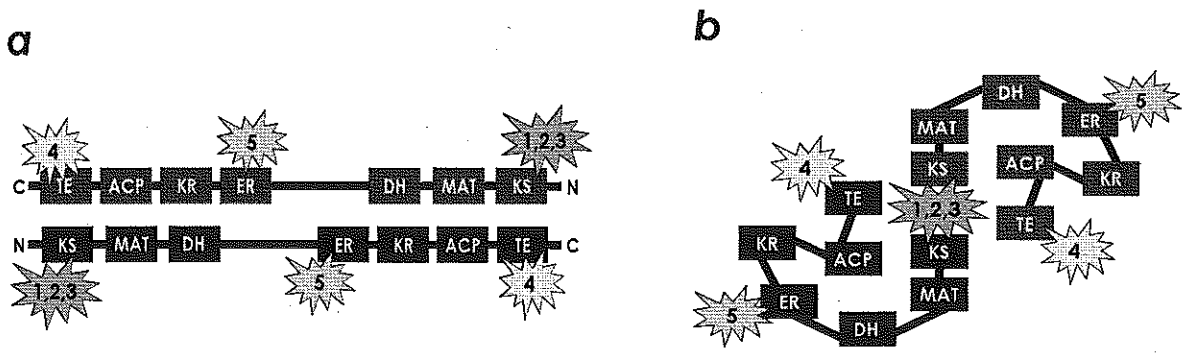
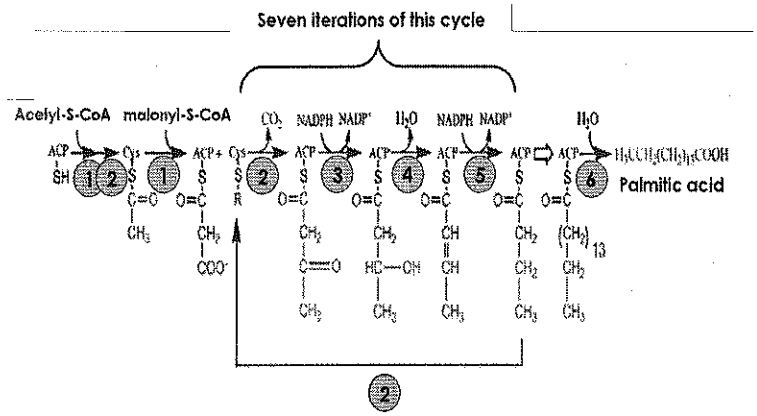


Fig. (1). Schematic representation of two models for organization of FASN enzymatic complex and target sites of chemical FASN blockers. *a.* In the conventional model, FASN enzymatic complex contains seven separate enzymatic pockets as a head-to-tail dimer with the KS and MAT domains of one monomer working together with the DE, ER, KR, ACP, and TE domains of the adjacent monomer. That is, two FASN monomers in the homodimeric form of the enzyme are arranged in a fully extended antiparallel orientation allowing functional interactions across the monomer interface. This conventional model for organization of FASN is largely based on the observation that the bifunctional reagent 1,3-dibromopropanone (DBP) is able to cross-link the active site cysteine thiol of the KS domain in one FASN monomer with the phosphopantetheine prosthetic group of the ACP domain in the other monomer. *b.* A revised model for FASN organization has been proposed by Asturias *et al.*. This alternative model predicts that the KS and MAT domains of both monomers lie closer to the center of the FASN dimer, where they can access the ACP of either subunit. Maier *et al.* recently revealed the architecture of mammalian FASN at 4.5 Å resolution, which fundamentally agrees with the Asturias´ revised model and demonstrate that mammalian FASN is, indeed, an intertwined dimer with a large dimerization interface running through the body of the molecule, perpendicular to the interfaced proposed in the classical scheme. **FASN blockers: 1:** Cerulenin; **2:** C75; **3:** EGCG and other naturally occurring polyphenolic compounds; **4:** Orlistat and other β-lactones; **5:** Triclosan.

A malonyl group derived from malonyl-CoA is condensed with an acetyl group from acetyl-CoA. The resultant β-ketoacyl derivative is reduced in three consecutive steps, β-ketoacyl reduction, β-hydroxyacyl dehydration and enoyl reduction, to the saturated acyl derivative, which then acts as a primer for further elongation and reduction cycles to yield ultimately a palmitoyl derivative. The latter is hydrolyzed by TE to free palmitate ["Fig. (2)"].

Fig. (2). Schematic diagram showing enzymatic steps involved in FASN-catalyzed endogenous FA biosynthesis that leads to palmitic acid. FASN is comprised of six enzymatic domains and an acyl-carrier protein (ACP). The steps in FA biosynthesis are as follows. (*i*) The MAT domain (1) transfers an acetyl group onto the ACP. It is then translocated to the active-site cysteine by KS (2). This position also serves as the loading position for the growing acyl chain in subsequent interactions. (*ii*) The MAT domain (1) then transfers a second malonyl group to the ACP, and the two are condensed (2) into a four-carbon product bound to the enzyme through the thiol group of the ACP. (*iii*) The KR (3) reduces the ketone at C-3 to an alcohol. (*iv*) The DH (4) further reduces the alcohol to an alkene. (*v*) The ER (5) further reduces the alkene bond to an alkane, and the ACP-bound chain is translocated back to the active-site cysteine (2). Steps *ii-iv* are then repeated six times to yield a 16-carbon, fully saturated palmitic acid bound to the ACP. (*vi*) The palmitate is released from FASN by the enzyme's intrinsic TE (6).



Since the FASN substrate malonyl-CoA, in addition to functioning as the carbon source for FASN-catalyzed fatty acid synthesis, is now recognized as an important signaling molecule involved in metabolic fuel sensing and appetite control, inhibitors of FASN activity inducing elevated malonyl-CoA levels represent a promising class of reagents for the treatment of obesity and obesity-related diseases, including diabetes and cardiovascular disorders. Indeed, some FASN inhibitors have shown potential for weight reduction in animal models, though their exact mechanism of action is until under discussion. Moreover, FASN blockade, either with naturally occurring or semi-synthetic chemical inhibitors or by RNA interference, induces apoptosis in cancer cells, making FASN a valuable target for the development of anticancer chemotherapeutical agents. Of note, in the last two years more than 50 papers have been published in the field of tumoral lipogenesis. Here, we will overview our current perspective on the use of FASN blockers as attractive agents for antineoplastic intervention.

FASN in normal and tumor cells: A large therapeutic index for cancer treatment

Fatty acid biosynthesis occurs in all living organisms and provides essential constituents of biological membranes, energy storage compounds and messenger substances. Furthermore, endogenous synthesized fatty acids act as post-translational protein modifiers and modulate gene expression. In humans, FASN, the sole enzyme capable of the reductive *de novo* synthesis of long-chain fatty acids from acetyl-CoA, malonyl-CoA, and NADPH, is highly expressed in liver, adipose tissue, and lactating mammary gland. Nevertheless, in adults FASN has been considered an enzyme of minor importance since exogenous saturated fatty acids are abundantly available through diet. That is, FASN activity in normal cells and tissues is significantly down-regulated and compensated with dietary fatty acids. Interestingly, extremely high levels of FASN are expressed in pre-malignant, invasive and metastatic lesions of many human epithelial cancers. This difference in expression and activity of FASN between normal and tumors cells provides an attractive approach to cancer therapy having the potential for a large therapeutic index. Indeed, the tumoricidal activity of chemical FASN inhibitors (**BOX 1**) has begun to emerge. Most of these FASN blockers have been employed in our experimental approach.

BOX 1. FASN inhibitors: A new therapeutic opportunity in cancer prevention and treatment

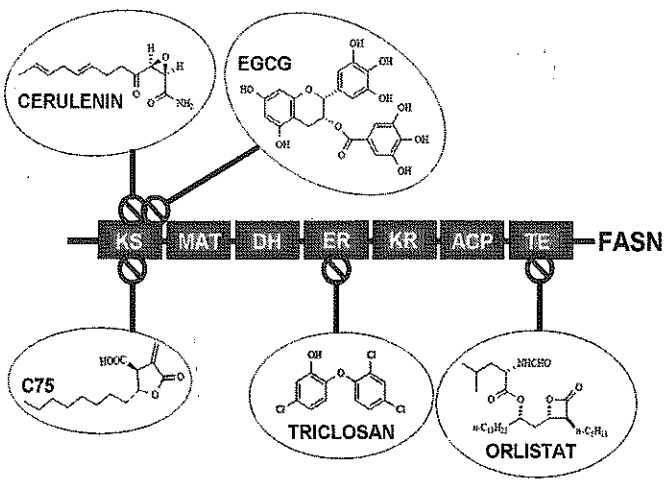
Cerulenin. Cerulenin [(2R, 3S), 2-3-epoxy-4-oxo-7, 10-*trans*, *trans*-dodecadienamide], a natural product derived from the fungus *Cephalosporum caerulens*, has been known since the 1960s as a potent non-competitive inhibitor of FASN-catalyzed FA synthesis. Cerulenin irreversibly inhibits FASN by binding covalently to the active cysteine site of the KS moiety, which performs the condensation reaction between the elongating FA chain and each successive acetyl or malonyl residue, thus causing complete FASN inactivation. Cerulenin-induced FASN blockade leads to selective cytotoxicity of tumour cells *in vitro* by triggering apoptosis, and it results in significantly delayed disease progression and in increased survival in human cancer xenografts. The clinical relevance of cerulenin is limited because of the chemical instability due to its very reactive epoxy group that interacts with other proteins and affects processes other than FASN activity, including palmitoylation, cholesterol synthesis and/or proteolysis.

C75. C75 (*trans*-4-carboxy-5-octyl-3-methylenebutyrolactone) is a novel cerulenin-derived semi-synthetic FASN inhibitor lacking the reactive epoxide present in cerulenin structure. C75 inhibits purified mammalian FASN activity with characteristics of a slow-binding inhibitor and, similarly to cerulenin, induces cytostatic and cytotoxic effects in cultured cells by blocking the KS domain of FASN. C75 has been shown to induce significant anti-tumour activity in human breast, endometrial, prostate, ovary and mesothelioma cancer cell lines and xenografts. While concomitantly inhibiting FA synthesis in tumour and normal liver, C75 treatments did not induce adverse effects on other proliferating cellular compartments such as bone marrow, gastrointestinal tract, skin or lymphoid tissues. Unfortunately, C75 treatment

robustly induces rapid and profou... weight loss and loss of adipose mass ... affecting food intake and energy expenditure, thus precluding its development as anti-tumour agent in the clinical setting.

Epigallocatechin-3-gallate (EGCG) and other naturally occurring polyphenols. The green tea component EGCG has been shown to act, similarly to cerulenin and C75, as a potent inhibitor of FASN activity through a competitive inhibition of NADPH for the same binding site in the KS domain of FASN. High levels of FASN activity in tumour cells can be dose-dependently inhibited by EGCG and this inhibition parallels decreased endogenous FA synthesis, inhibition of cancer cell growth and induction of apoptosis. Conversely, EGCG treatment of non-malignant cells exhibiting low levels of FASN activity leads to a decrease in growth rate but not to induction of apoptosis. Other EGCG-related naturally occurring polyphenols (*i.e.*, the flavonoids luteolin, quercetin and kaempferol) also inhibit the activity of purified FASN, and these anti-FASN effects strongly associated with the ability of polyphenolic compounds to arrest cell growth and induce apoptosis in prostate and breast cancer cells.

Orlistat. Unlike cerulenin, C75 and EGCG, all of them inhibiting the KS domain of FASN, the β-lactone orlistat (tetrahydrolipstatin; Xenical®, an US Food and Drug Administration-approved anti-obesity drug) elicits its anti-FASN effect by blocking the TE domain, which is responsible for releasing the end-product palmitate from the ACP of the enzyme. Specifically, orlistat inhibits FASN activity in intact cells with no effect on the abundance of FASN protein but rather acting as a tight-binding irreversible inhibitor of the serine hydrolase activity within the TE domain of FASN. Orlistat-induced FASN blockade has demonstrated potent anti-proliferative and pro-apoptotic effects against cultured prostate, breast, colon, stomach and ovarian cancer cells, with no effect on normal cells. Although orlistat has successfully been tested in xenografts mouse models, preventing the growth of prostate tumours without outward signs of toxicity, it possesses poor solubility and extremely low oral bioavailability. Therefore, a novel formulation will be required for treating tumours other than those confined to the gastrointestinal tract.



Triclosan. Triclosan (2,4,4-trichloro-2-hydroxydiphenyl ether), an antibiotic widely used for over 30 years as an antibiotic in soaps mouthwashes and other oral dentifrices, has been shown to block FASN activity by specifically inhibiting the ER domain of the FASN multi-enzyme complex. Biologically relevant dose levels of triclosan significantly inhibit growth and reduce the cell viability of breast cancer cells in culture and suppress rat mammary carcinogenesis. Triclosan ER-targeted domain could be particularly attractive for the development of novel anti-FASN metabolites as inhibition at this site will increase the concentration of the enoyl thiolester intermediate, which strikingly resembles CM-55 (a synthetic analogue of cerulenin) and C75. If triclosan-induced accumulation of this FASN intermediate further leads to the "side-inhibition" of the KS domain remains to be elucidated.

Molecular mechanisms underlying anti-tumoral actions of FASN inhibitors

The fact that FASN blockade, either with naturally occurring or chemical inhibitors or by RNA interference, significantly attenuates the growth and proliferation of tumour cells strongly suggests that tumour-associated FASN hyperactivity is vital for the survival of cancer cells. However, it has been unresolved the ultimate mechanisms underlying the cytostatic, cytotoxic as well as apoptotic effects occurring upon FASN inhibition in cancer cells. Several mechanisms have been proposed:

- **End-product starvation:** Following specific inhibition of FASN activity in cultured human cancer cells, a rapid decline occurs in endogenous FA synthesis, with subsequent cell cycle arrest that culminates in apoptotic cell death. Although FASN blockade-related cell death commonly occurs despite the presence of exogenous FAs in the culture medium provided by foetal bovine serum, it has been possible to rescue the cytotoxic effects of chemical FASN inhibitors on certain tumour cells cultured in FA-free culture conditions by the addition of high concentrations of palmitate (*i.e.*, the primary end-product of FASN) or oleic acid (*i.e.*, a FA synthesized from palmitate and one of the most abundant circulating FA). Conversely, exogenous addition of supra-physiological concentrations of non-FASN related FAs (*e.g.*,

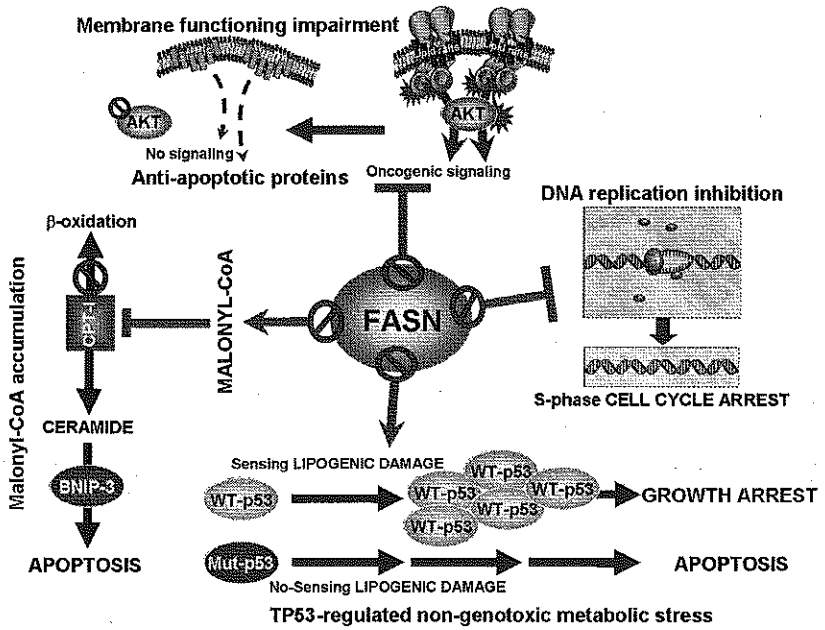
α-linolenic, docosahexaenoic, eicosapentaneoic, linoleic or arachidonic FAs) fail to reverse FASN inhibition' effects on cancer cell viability. Therefore, FASN-driven lipogenesis may be not essential for high cell proliferation when a sufficient amount of exogenous FAs is present in the microenvironment. This strongly suggest that, in highly dividing tumour cells, exacerbated synthesis of FAs may provide a large pool of available lipids required for the newly synthesized cellular membrane. Considering that tumour-associated FASN plays a major role in the synthesis of phospholipids, a good molecular candidate explaining the "end-product starvation" hypothesis is phosphatidylcholine, the most abundant lipid affected by modulation of FASN activity. This assumption that lipid synthesis *per se* may be important for cancer biology is supported by the fact that equivalent tumoricidal effects can be observed when targeting FASN and acetyl-CoA carboxylase-α (ACACA), both governing *de novo* lipogenesis. In agreement with this assumption, inhibition of choline kinase, an oncogenic enzyme involved in the *de novo* synthesis of phosphatidylcholine, similarly induces apoptotic cell death selectively in cancer cells.

- **Disturbance of membrane functioning:** In contrast to exogenously-derived (dietary) FAs, the newly FASN-synthesized FAs are saturated or at most monounsaturated. Therefore, exacerbated lipogenesis in tumour cells may not only evoke quantitative changes in cellular membranes required for cell growth as describe above, but may also changes the lipid composition of membranes. Supporting this notion, it has recently been demonstrated that tumour-associated FASN activity is mainly involved in the production of phospholipids partitioning into detergent-resistant membrane microdomains (lipid raft-aggregates). Raft-aggregates, which are defined as plasma membrane domains enriched with glycosphingolipids and cholesterol that render them insoluble to non-ionic detergents, are implicated in key biological processes including signal transduction, intracellular trafficking, cell polarization and cell migration. Many surface receptors are constitutively or inducibly associated with lipid rafts, and it has been suggested that rafts function as platforms regulating the induction of signalling pathways. For instance, clusters of the ERBB2-coded $p185^{ERBB2}$ oncoprotein, a key oncogenic transducer in many types of human cancers, co-localize with lipid rafts and the lipid environment the cell membrane of tumour cells profoundly influences the association properties and biological function of $p185^{ERBB2}$. Lipid rafts also regulate the signalling from EGFR (ERBB1), presumably by altering its kinase activity and changing its association state with membrane proteins. Therefore, it is likely that FASN inhibition, by inducing major changes in the synthesis of membrane phospholipids and assembling of lipid rafts, will impair a correct localization and/or functioning of members of the ERBB tyrosine kinase receptor network at the cellular membrane of tumour cells. For instance, FASN blockade may induce a shift in the equilibrium between transport of ERBB receptor to and from the membrane favouring an increased ERBB internalization followed by intracellular degradation. In agreement with this hypothesis, pharmacological inhibition of FASN activity concomitantly and equally reduces the expression of cell surface-associated $p185^{ERBB2}$ and of the glycosphingolipid GM1, a lipid raft marker. Similarly, FASN blockade has recently been found to disrupt the proper display and subsequent cell membrane association between the Vascular Endothelial Growth Factor (VEGF) receptor (VEGFR2/KDR/Flk1) and Gαq11, a member of the heterotrimeric G protein family that is required for KDR signalling. While a precise mechanism for FASN inhibition-induced improper cell membrane trafficking will require further studies, these findings likely relate either to the inability of cells to assemble lipid rafts or to the disruption of protein palmitoylation (a posttranslational modification allowing key signalling proteins to attach to the plasma membrane) upon FASN blockade.

- **Inhibition of DNA replication (S-phase cell cycle cell arrest):** FASN blockade produces rapid, potent inhibition of DNA replication, leading to a block in the cell cycle before G_1. Indeed, there are data that support an S-phase arrest in breast, colon and glioma cancer cells. Although the molecular mechanism(s) of FASN-inhibition induced cell cycle arrest is still no clear (among others, p53-$p21^{WAF1-CIP1}$, BRCA1, Skp2, $p27^{Kip1}$, and NF-kappaB may be involved), most of the FAs produced by tumour cells are incorporated into membrane phospholipids, and phospholipid synthesis is inhibited when FA synthesis is inhibited. Phospholipid biosynthesis is greatest during G_1 and S phases, with doubling of the membrane mass occurring during S-phase in preparation for cell division. Indeed, most of the phospholipid accumulation in preparation for mitosis takes place during S-phase. Thus, cells in S-phase should be more sensitive to changes en FASN-driven phospholipid metabolism.

- **TP53-regulated non-genotoxic metabolic stress:** The decision between apoptosis and growth arrest following FASN inhibition is greatly influenced by the status of TP53. Thus, FASN inhibitors have been shown to be more effective in initiating apoptosis in cancer cells with non-functioning TP53, whereas tumour cells with intact TP53 function tend to be exhibit a cytostatic response. Cancer cells rendered TP53 deficient by either constitutive expression of the human papilloma virus 16 E6 genes or by performing loss-of-function genetic screens with TP53 RNAi interference-based approaches, undergo extensive apoptosis after FASN inhibition. Since FASN-induced accumulation of TP53 is not related to direct damage effects on DNA, these results, altogether, indicate that TP53 protein accumulation actively limits the apoptotic effects upon metabolic stress induced by FASN inhibition. The recent discovery that the FASN gene is a conserved TP53 family target from worm to human, not only reveals a functional link between these two proteins

throughout the evolution but further support the notion that TP53 is a novel molecular sensor of energy imbalance occurring upon perturbation of endogenous FA metabolism in tumour cells.



- **Toxic accumulation of malonyl-CoA:** Cell death resulting from the blockade of tumour-associated FASN may be metabolic in origin and occurs due to malonyl-CoA-induced inhibition of FA β-oxidation. Malonyl-CoA not only is an intermediate in the *de novo* synthesis of FAs but also a physiological inhibitor of CPT-1, the enzyme that governs the transfer of long-chain fatty acyl CoA molecules from the cytosol into mitochondria were they are oxidized. Because of the latter action, a decrease in the concentration of malonyl-CoA results typically in an increase of FA oxidation, whereas an increase in malonyl-CoA has the opposite effect. It is therefore plausible that FASN inhibition that causes malonyl-CoA accumulation (as would be expected from the fact that FASN uses malonyl-CoA as a substrate) may lead to concomitant inhibition of CPT-1, thus triggering a cellular energetic crisis and apoptosis. But, how the supraphysiologic levels of malonyl-CoA leads to apoptosis in tumour cells? It has recently been demonstrated that, upon specific FASN blockade, malonyl-CoA-induced inhibition of CPT-1 promotes the accumulation of ceramide (a sphingolipid that has been implicated in apoptotic response of cells to death inducers) followed by induction of the proapoptotic genes *BNIP3*, *TRAIL* and *DAPK2*, all of them involved in ceramide-mediated apoptotic pathway.

- **Inhibition of anti-apoptotic proteins:** Pharmacological inhibition of the pro-survival and anti-apoptotic PI-3'K/AKT pathway has been shown to synergistically interacts with FASN blockade to enhance tumour cell death *via* activation of caspases and down-regulation of anti-apoptotic proteins, including XIAP, cIAP-1 and AKT. On the other hand, inhibition of FASN activity results in the down-regulation of active AKT, which precedes the induction of tumour cell apoptosis *in vitro* and *in vivo*, suggesting a potential mechanism for FASN inhibition-related cell death. Therefore, downstream of GFR, FASN inhibition appears to provoke down-regulation of the PI-3'K/AKT transduction pathway, while constitutive activation of AKT protects against FASN inhibition-induced cell death.

Molecular markers for sensitivity to FASN inhibitors

Why inhibition of FASN-catalyzed endogenous fatty acid biogenesis results in specific apoptotic cell death of cancer cells remains an area of active investigation. Nevertheless, the activity of a number of pathways appears to modulate the tumoricidal effects of FASN inhibition.

- **Nonfunctioning p53.** As described above, FASN inhibition has been shown to be more effective in initiating apoptosis in cancer cells with nonfunctioning p53, whereas tumor cells with intact p53 function tend to exhibit a cytostatic response. Although the first and most extensively studied function described for the tumor suppressor protein p53 was the induction of growth arrest and apoptosis after DNA damage, important roles for p53 have been recognized in the cellular responses to a variety of non-genotoxic metabolic stresses, including hypoxia, acidosis, and perturbations of RNA and protein synthesis. Interestingly, pharmacological inhibition of FASN activity has recently been shown to produce both cytostatic and cytotoxic effects modulated by p53 in cancer cells. Thus, FASN blockade produces rapid, potent inhibition of DNA replication and S-phase progression, and induces accumulation of p53 in colon and breast cancer cells. Although these findings suggest that

perturbation of FASN activity is a novel cellular signaling belonging on the list of metabolic stresses regulated by p53 in cancer cells, it could be also argue that DNA damage might be occurring, either as a direct effect of chemical FASN inhibitors on DNA molecule, or as a downstream effect of FASN inhibition. However, several other observations argue against FASN inhibition-induced DNA damage. First, Li *et al.* detected no DNA damage in FASN inhibitors-treated cancer cells by using the very sensitive in detecting low levels of DNA damage single cell gel electrophoresis (comet) screening assay. Second, as described above, FASN inhibitors-induced toxicity in tumor cells appears to be modulated by alterations in pathway activity and/or substrate levels. Finally, the toxic effects of chemical FASN inhibitors are largely dependent on their ability to inhibit FASN as breast cancer cells became insensitive to C75-induced cytotoxicity when FASN gene expression was specifically silenced by RNAi. These observations together demonstrate that inhibition of FASN-inhibition-induced accumulation of p53 is not related to direct effects of chemical FASN blockers on DNA, thus confirming the important role for p53 in a non-genotoxic metabolic stress such as blockade of endogenous FA metabolism.

- The decision between apoptosis and growth arrest following FASN inhibition is greatly influenced by the status of p53. First, loss of p53 function substantially increases the sensitivity of tumor cells to chemical FASN inhibitors. A large, dose-dependent increase in apoptosis was observed in FASN inhibited RKO colon cancer cells rendered p53-mutant by stable transfection with a dominant-negative mutant p53 gene. Similarly, MCF-7 breast cancer cells rendered p53-deficient by constitutive expression of the human papilloma virus 16 *E6* gene underwent extensive apoptosis within 24 h after FASN inhibition. Accordingly, we recently characterized wild-type p53 as a key molecular component of FASN-related cellular signaling by using RNA interference (RNAi)-based approach, a powerful new tool with which to perform loss-of-function genetic screens. p53 protein levels remained unchanged in C75-treated MCF-7 breast cancer cells transiently transfected with a pool of sequence-specific double-stranded RNA oligonucleotides targeting p53 gene when compared to p53 up-regulation observed in control cells. Importantly, RNAi-induced silencing of p53 expression together with C75-induced FASN blockade resulted in an enhancement of apoptosis that was significantly higher than the additive value of the two treatments alone. Thus, siRNA p53 and C75 combined caused 4 times more apoptotic cell death than C75 alone, and up to 30 times more apoptotic cell death than siRNA p53 alone. These results, altogether, indicate that FASN inhibition triggers p53 protein accumulation, which actively limits the apoptotic effects upon metabolic stress induced by FAS inhibition. Strikingly, there was no apparent relationship between the p53 mutational status and sensitivity to chemical FAS inhibitor in a panel of human breast cancer cell lines. However, the degree of p53 mRNA expression was predictive of sensitivity to C75-induced cytotoxicity, with low-TP53 mRNA expressing breast cancer cells showing hypersensitivity to FAS blockade. These findings strongly suggest that:

  a.) p53 is a novel molecular sensor of energy imbalance after the perturbation of endogenous fatty acid metabolism in breast cancer cells.
  b.) p53 function closely influences the decision between apoptosis and growth arrest following FASN blockade; and
  c.) pharmacological inhibitors of FASN activity may be clinically useful against breast carcinomas exhibiting mutation or aberrant expression of p53.

- **Overexpression of the Her-2/*neu* (*erb*B-2) oncogene.** Her-2/*neu* overexpression has also been linked to FASN inhibition-induced cytotoxicity. Her-2/*neu* (also called *neu* and *erb*B-2) oncogene codes for the transmembrane tyrosine kinase orphan receptor $p185^{Her\text{-}2/neu}$ and, at

present, represent one of the most important oncogenes in the etiology, progression, and chemosensitivity of various types of human malignancies. Using a wide panel of human breast cancer cell lines we previously described that high levels of both FASN protein and FASN enzymatic activity positively correlate with Her-2/*neu* gene amplification and/or Her-2/*neu* protein overexpression, whereas low to undetectable levels of FASN correlates with low to undetectable levels. This molecular connection between Her-2/*neu* and FASN was further confirmed by Kumar-Sinha *et al.* To focus on genes specifically affected by Her-2/*neu*, they made use of a human mammary epithelial cell line, H16N2, which ectopically overexpress Her-2/*neu*. In addition, they examined a panel of breast cancer cell lines recently derived from patients (*e.g.*, SUM149). Using DNA microarrays, they defined a select set of genes induced by Her-2/*neu* overexpression and concomitantly repressed by Her-2/*neu* inhibitors. One of the Her-2/*neu*-transcriptionally regulated genes they identified was FASN. Moreover, pharmacological inhibition of FASN activity preferentially induced apoptotic cell death of breast epithelial cells engineered to overexpress Her-2/*neu* relative to matched control vector cells. Accordingly, we also observed that normal murine fibroblasts NIH-3T3 and non-cancerous breast epithelial MCF10A cells engineered to overexpress Her-2/*neu* similarly exhibited a significant up-regulation of FASN gene and protein expression, while pharmacological inhibition of FASN activity preferentially induced apoptotic cell death of Her-2/*neu*-transformed cells relative to untransformed controls.

Although these findings strongly suggested that an active FASN-driven cellular signaling is necessary for Her-2/*neu*-enhanced cell survival, it was reasonable to suggest that growth factor receptors other than Her-2/*neu* may also regulate FASN levels in transformed cells and, hence, the degree of cancer cell sensitivity to FASN blockers. To evaluate this possibility and because the function of Her-2/*neu* is closely linked to that of its family co-members EGFR (*erb*B-1), Her-3 (*erb*B-3), and Her-4 (*erb*B-4), we characterized the relationship between the cytotoxic effects of the chemical FASN blockers cerulenin and C75 and the expression levels of the Her-1/-3/-4 in a panel of cancer cell lines naturally or ectopically expressing varying amounts of Her-2/*neu*. Cancer cell lines were arbitrarily defined as "low-sensitive" and "highly-sensitivity" on the basis of their IC_{50} values (*i.e.*, the concentration of drugs producing 50% reductions in cell viability) for cerulenin and C75. Interestingly, a striking picture emerged when the two groups of breast cancer cells were plotted against Her-1/-2/-3/-4 expression levels. There was no apparent correlation between the expression of Her-1, Her-3, or Her-4 with the sensitivity to cerulenin and C75. However, all the breast cancer cell lines exhibiting "low-sensitivity" to chemical FASN blockers were low-Her-2/*neu*-expressors, while all the breast cancer cell lines exhibiting "high-sensitivity" were Her-2/*neu* overexpressors. Although this analysis clearly established that there exists a linear correlation between Her-2/*neu* expression and the cytotoxic effects of FASN blockers, it could be argued that the cytotoxic effects of chemical FASN blockers correlate, in fact, with constitutive FASN expression levels. However, Her-2/*neu*-overexpressing BT-474 and MDA-MB-453 breast cancer cell exhibited cerulenin and C75 IC_{50} values comparable to those found in SK-Br3 breast cancer cells, a unique paradigm of FASN-overexpressing breast cancer cells in which FAS enzyme constitutes up to 28%, by weight, of the cytosolic proteins. These results revealed that the possession of high levels of Her-2/*neu* oncogene, rather than FASN overexpression, may be a molecular determinant for hypersensitivity to FASN blockers-induced cytotoxicity in human breast cancer cells. To conclusively confirm the selectivity of chemical inhibitors towards Her-2/*neu* overexpressors compared with low-Her-2/*neu*-expressing breast cancer cells, we analyzed the growth inhibitory effects of cerulenin and C75 following the forced expression of Her-2/*neu* oncogene in MDA-MB-231 breast cancer cells, which naturally

express very low amounts of both Her-2/*neu* and FASN. In this scenario, we demonstrated that the transfection with the full-length cDNA of the human Her-2/*neu* oncogene results in an enhancement of FASN expression that does not reach the levels of FAS in other naturally Her-2/*neu*-overexpressing model such as SK-Br3 breast cancer cells. MDA-MB-231/Her-2 cells were completely inhibited by FASN blockers in their capacity to form colonies in soft-agar, whereas pharmacological inhibition of FASN activity had no significant effects on the anchorage-independent growth of Her-2/*neu*-negative MDA-MB-231 cells. Moreover, TUNEL staining of C75-treated MDA-MB-231/Her-2 cells revealed a noteworthy increase in the number of cell dying from apoptosis, whereas no major signs of apoptosis were observed in Her-2/*neu*-negative MDA-MB-231 breast cancer cells. Therefore, the biological consequences of FASN inhibition on malignant transformation, cancer cell proliferation and cancer cell survival growth appear to truly depend, at least in part, on the expression levels of Her-2/*neu* oncogene [18]. Importantly, FASN expression has recently been associated with Her-2/*neu* expression in clinical specimens from aggressive breast cancers.

- **Hyperactivation of the PI-3'K downstream effector Protein Kinase B (AKT).** In view of the experimental data indicating that in human cancer cells, overexpression of FASN can be largely attributed to constitutive activation of the PI-3'K/AKT kinase pathway, Van de Sande *et al.* examined the activation status of the AKT pathway, and whether this activation coincided with increased FASN expression in clinical prostate cancer tissues. Their data strongly suggested that high-level expression of FASN in prostate cancer is linked to phosphorylation and nuclear accumulation of activated AKT. Moreover, the specific inhibition of FASN gene by siRNA leads to apoptosis of prostate tumor cells, and inhibition of the PI-3'K-kinase pathway synergizes with FASN siRNA to enhance tumor cell death. These results, altogether, provide a strong rationale for exploring the therapeutic use of an inhibitor of the PI-3'K/AKT signaling pathway in conjunction with FASN blockers to inhibit cancer cell growth. In this regard, we observed that combined treatment of the FASN blocker C75 and the anti-mitotic drug paclitaxel (Taxol®) inactivated the anti-apoptotic AKT kinase more than either agent alone, as evidenced by a synergistic down-regulation of AKT phosphorylation at its activating site Ser(473) without affecting AKT protein total levels in breast cancer-derived cell lines.

In a recent study of human ovarian cancer cells, FASN and activated Protein Kinase B (P-AKT) expression were coordinately regulated, suggesting a potential mechanism for FASN inhibitors-related apoptosis. Treatment of human ovarian cancer cells harboring constitutively active AKT (P-AKT) with the PI-3'K inhibitor LY294002 abolished P-AKT activity and potentiated apoptosis induced by FASN inhibitors, cerulenin or C75. Furthermore, inhibition of FASN activity resulted in the down-regulation of P-AKT, which preceded the induction of apoptosis both *in vitro* and *in vivo*. We similarly observed that treatment of SK-Br3 human breast cancer cells, which exhibit Her-2/*neu*-driven constitutive hyperactivation of the PI-3'K/AKT transduction cascade, with the PI-3'K inhibitor LY294002 abolished P-AKT activity and dramatically enhanced apoptosis induced by FASN inhibitors such as C75 or orlistat. Similarly, Liu *et al.* recently reported that LY294002-induced inhibition of the PI-3'K/AKT pathway synergistically sensitized EGFR-overexpressing MDA-MB-468 human breast cancer cells to cerulenin-induced apoptosis *via* activation of caspases, down-regulation of anti-apoptotic proteins, including XIAP, cIAP-1 and AKT, and possibly, activation of Bak in mitochondria.

Collectively, the above results are consistent with a working model in which AKT activation regulates FASN expression, at least in part, whereas FASN activity modulates AKT

activation. Indeed, the experimental findings indicate that the molecular mechanisms of specific FASN inhibition appear to provoke down-regulation of the *Her* receptors → PI-3'K → AKT signal transduction pathway, while constitutive activation of AKT protects against FASN inhibitors-induced cell death.

FASN and Cytotoxics

Given the widespread expression of FASN in many human cancers, the differential expression of FASN between cancer and normal cells, it is reasonable to suggest that combinations of novel compounds directed against FASN-dependent endogenous FA biosynthesis with currently used cytotoxic drugs (*e.g.*, anthracyclines, platinum derivatives, anti-metabolites, microtubule-interfering agents) and selective modulators of the biological response (*e.g.*, monoclonal antibodies) might provide increased efficacy over existing therapy in human carcinomas.

FASN and Anthracyclines

Anthracyclines (*e.g.*, doxorubicin, daunorubicin, epirubicin, idarubicin) have been widely used over the last 20 years and are firmly established as effective, first-line chemotherapeutic agents in cancer disease. Anthracyclines-induced cytotoxicity can be attributed to several mechanisms related to their chemical structure. A polycyclic chromofore moiety allows anthracyclines to intercalate between DNA base pairs further blocking the activity of the Topoisomerase II-α (TOP2A) enzyme. On the other hand, a quinone ring moiety allows anthracycline to generate highly reactive oxygen species (ROS) that, in the presence of water, can produce superoxide anions capable to induce cellular damage related to interactions with DNA, proteins and lipids.

Mathematical assessment of the nature of cytotoxic interactions between FASN blockade and anthracyclines. A preliminary study investigating the effects of cerulenin-induced FASN blockade in the efficacy of the anthracycline doxorubicin (DOX) revealed that mainly additive cytotoxic interactions occurred when the chemical FASN blocker and DOX were combined upon three different schedules of treatment (*i.e.*, FASN inhibition + DOX, FASN inhibition → DOX, DOX → FASN inhibition) in FASN-overexpressing SKBR3 cells. A statistically significant supra-additive (*i.e.*, synergistic) interaction was observed when MCF-7 cells -which naturally express moderate amounts of endogenous FASN- were concurrently exposed to cerulenin and DOX. However, this synergism was loss when DOX was administered before the FASN blocker in MCF-7 cells.

Since a major barrier to effective DOX-based treatment of human malignancies is the multifactorial development of multi-drug resistance (MDR) by tumor cells -which is partially dependent on the overexpression of the transmembrane transport glycoprotein pump $p170^{mdr\text{-}1}$ to cause efflux of some anticancer drugs from cells- our group further investigated the sensitizing effects of pharmacological FASN inhibition in anthracycline resistant- and $p170^{mdr\text{-}1}$-overexpressing NCI/ADR-RES cells. Remarkably, co-exposure to cerulenin increased DOX cytotoxicity up to 39 times while a dramatic 80-fold increase in the sensitivity of NCI/ADR-RES cells to DOX was observed when the cerulenin followed by DOX regimen was used. Moreover, when the nature of the interaction between FASN inhibition and DOX was assessed using the isobologram method, a strong synergism was observed regardless the schedule of treatment.

The above mentioned findings strongly suggested that differential synergistic interactions between FASN inhibition and DOX-induced cell injuries occur among anthracycline-resistant and

anthracycline-sensitive cancer cells. Recently, Liu *et al.* further confirmed that elevated FASN expression functionally contributes to increased drug resistance levels to ADR in MCF-7/AdVp3000 cells, which were derived from ADR-sensitive MCF-7 cells by stepwise selections using increasing concentrations of DOX in the presence of verapamil. During their search for potential candidates that were in part responsible for the drug resistance phenotype of MCF-7/AdVp3000 cells, they accidentally found that FASN was differentially overexpressed in MCF-7/AdVp3000 cells. To determine whether FASN overexpression caused DOX resistance, they first determined if the elevated FASN expression actively contributed to the drug resistance phenotype. For this purpose, FASN expression in MCF-7/AdVp3000 cells was knocked down using small interfering RNA (siRNA) targeting FASN.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and anthracyclines. FASN inhibition produces profound inhibition of DNA replication and S-phase progression in cancer cells, which suggests a direct linkage at a regulatory level between FASN-catalyzed endogenous FA biogenesis and DNA synthesis in proliferating tumor cells. In this regard, it is tempting to speculate that FASN blockade can also suppress DNA repair capacity, possibly contributing to the enhancement of the anti-tumor effect of DNA-damaging agents including DOX. Among the DNA repair/replication genes, *TOP2A*, a key enzyme in DNA replication and a molecular target for DOX that is located adjacent to the *HER2* oncogene at the chromosome location 17q12-q21 and is either amplified or deleted in almost 90% of HER2-amplified primary breast tumors. Because TOP2A activity is critical in various processes of DNA metabolism, including transcription, recombination, replication, and chromosome segregation during cell division, some authors have suggested that treatment with anti-HER2 antibodies such as trastuzumab (Herceptin™), by decreasing TOP2A expression, may enhance the chemosensitivity of breast cancer cells to TOP2A inhibitors such as DOX. Conversely, Harris *et al.* demonstrated that induction of TOP2A activity after HER2 activation is associated with a differential response to breast cancer chemotherapy. Indeed, their experiments showed that the cytotoxicity of DOX is inhibited in HER2 positive breast cancer cells by the anti-HER2 antibody trastuzumab, which was accompanied by a decrease in TOP2A protein and activity, suggesting that this is the mechanism of change in DOX response. Interestingly, we previously reported that pharmacological inhibition of FASN activity, similarly to functional blockade of HER2, significantly down-regulates TOP2A protein expression in HER2-overexpressing breast cancer cells. Accordingly, pharmacological inhibition of FASN activity in combination with DOX does not demonstrate synergism in HER2-overexpressing SKBR3 breast cancer cells. The fact that TOP2A inhibitors work in an epigenetic fashion on FASN expression by up-regulating the activity of the FASN gene promoter might counteract the appearance of antagonistic/protective interactions between chemical FASN blockers and DOX in breast cancer cells bearing *HER2* gene amplification.

Intracellular drug accumulation is a complex process that includes drug uptake into the cell, retention and distribution within the cell, and efflux from the cell. At any given time, the net uptake (accumulation) of a drug in cells is the difference between the amount of drug uptake and efflux. $p170^{mdr1}$-mediated drug efflux decreases intracellular net drug uptake, and it causes cells to be drug-resistant. Therefore, it would be reasonable to suggest that the enhanced DOX efficacy observed upon FASN blockade in DOX-resistant breast cancer cell lines might relate to either enhanced intracellular DOX uptake by FASN inhibition-induced alteration in drug accumulation due to changes in cell membrane fluidity/permeability and/or to the suppression of $p170^{mdr-1}$ expression/activity. When the effects of the FASN inhibition on DOX net uptake and efflux were assessed by monitoring the intracellular accumulation of auto-fluorescent DOX in NCI/ADR-RES cells no significant differences

in DOX accumulation kinetics, as assessed by flow cytometry, were observed in the absence or presence of cerulenin. Therefore, it is likely that FASN activity does not affect $p170^{mdr-1}$ transporter activity. Accordingly, Liu *et al.* recently demonstrated that knocking down FASN expression does not affect drug accumulation in MCF-7/AdVp3000 cells that overexpress ABCG2, a member of the ABC transporter family, thus indicating that FASN blockade does not affect the ABC transporter activity in these cells.

It has been shown that DOX induces apoptosis in MCF-7 cells by generating sphingosine from ceramide. Recently, it was also found that knocking down of FASN expression in breast cancer cells using siRNA significantly up-regulated the expression of several pro-apoptotic genes, such as BNIP3, TRAILS, and DAPK2, in addition to ceramide. It is reasonable to suggest that altered FASN activity/expression levels would affect the level of *de novo* biosynthesis of cellular lipids to protect breast cancer cells from drug-induced apoptosis. Interestingly, the simultaneous presence of the main metabolic end-product of FASN palmitate significantly reversed the potentiating effects of FASN inhibition on DOX-induced cytotoxicity in NCI/ADR-RES cells, thus suggesting that the synergistic interaction between chemical FASN inhibitors and DOX may result, at least in part, from FASN product depletion. Accordingly, Liu *et al.* recently reported that simply increasing the cellular level of palmitate by exogenous supplementation significantly increased the level of DOX resistance in MCF-7 cells.

Several reports suggest that DOX-induced apoptosis is linked to formation of reactive oxygen species (ROS) derived from redox activation of DOX. DOX has been reported to induce ROS generation in various tumor cells and several groups have reported that inhibiting DOX-induced intracellular oxidative stress made tumor cells more sensitive to DOX. Since it has been shown that depletion of the cellular pool of palmitic acid upon specific silencing of *FASN* gene is associated with induction of apoptosis concomitant with the formation of ROS, it is reasonable to suggest that specific suppression of FASN activity by pharmacological means would enhance tumor cell sensitivity to ROS-generating anticancer drugs such as DOX. Experiments are currently underway in our laboratory to resolve the relationship between FASN-regulated redox status and DOX efficacy. Interestingly, we recently observed that siRNA-induced specific suppression of Acetyl-CoA Carboxylase-alpha (ACCA) -a key regulatory step in the FA synthesis pathway- drastically enhances breast cancer cell sensitivity to DOX. Moreover, concurrent supplementation with N-acetylcysteine (NAC), a precursor of glutathion (GSH; the substrate of the ROS defense enzyme GSH peroxidase and the GSH transferase family of detoxification enzymes), severely impairs cerulenin-induced cytotoxicity and significantly decreases the ability of the FASN blocker to enhance breast cancer cell sensitivity to DOX. Taken together, these findings suggest that ROS play an important role in terms of the mechanism of action underlying FASN inhibition-induced enhancement of anthracyclines efficacy against breast cancer cells.

FASN and Microtubule-interfering agents

FASN and Paclitaxel (Taxol™)

Because chemotherapeutic regimens in advanced breast cancer frequently use microtubule inhibitors such as taxanes, we looked into the role of FASN activity on the cytotoxic activity of paclitaxel (Taxol™, Bristol-Myers Squibb Company, Princeton, NJ), a member of the taxanes class of anti-neoplastic agents that exhibits significant activity against metastatic breast cancer. Taxol™ binds to β-tubulin, stabilizes the microtubule, prevents its depolymerization, leads to arrest of cells in G_2-M and ultimately, it triggers apoptosis. Taking advantage of the well-known ability of C75 to specifically block FASN-dependent *de novo* fatty acid biosynthesis, we employed this γ-lactone as an

experimental tool to evaluate the role of FASN activity on breast cancer cell response to Taxol™-induced cell damage. Using a panel of human breast cancer cell lines differentially expressing FASN, we determined increases in overall cytotoxic effects, as compared to single-agent treatment alone. In an attempt to provide the preclinical rationale for the optimal clinical development of these combinations, isobologram and median-effect plot (Chou and Talalay) methods were used to asses the synergism or antagonism between C75 and Taxol™. The efficacy of different schedules of administration (simultaneous exposure to C75 and Taxol™ or sequential exposure to C75 followed by Taxol™) was also compared.

Mathematical assessment of the nature of cytotoxic interactions between FASN blockade and paclitaxel. Our studies demonstrated that the greatest number of synergistic combinations as well as the greatest magnitude of synergy occurred in FASN-overexpressing SK-Br3 cells, whereas low-FASN-expressing MDA-MB-231 demonstrated the lowest number of synergistic combinations as well as the lowest magnitude of synergy between C75 and Taxol™. Moreover, we observed that the nature of the cytotoxic interaction between C75 and Taxol™ in individual breast cancer cell lines was sequence-dependent. Thus, synergism was observed mainly when breast cancer cells were exposed to the 2 agents simultaneously, whereas additive or even antagonistic interactions were observed when chemical FAS blocker C75 preceded Taxol™. From a clinical perspective these findings suggest that the simultaneous administration of FASN inhibitors and Taxol™ may be the optimal schedule for the combination in terms of cytotoxic effects.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and paclitaxel. Although the ultimate molecular mechanisms of interaction operating with these schedules were not conclusively addressed by our experiments, these events were associated with several signaling interactions. In agreement with earlier studies, exposure to Taxol™ leaded to slight increases in the activation status of p38 MAPK and ERK1/ERK2 MAPK. Interestingly, p38 MAPK and ERK1/2 were also molecular targets in cells exposed to FAS inhibitor C75. Thus, it is reasonable to suggest a working model of signaling interactions in breast cancer cells exposed to C75 and Taxol™, in which the relative outputs of these pathways may influence the schedule-dependent synergistic cell death response to FAS inhibition. Co-exposure of Taxol™-treated cells to C75 synergistically activated p38 MAPK, induced further a dramatic accumulation of p53 phosphorylated at Ser46, a p38 MAPK-regulated pro-apoptotic modification of p53, and concomitantly decreased the level of active AKT and ERK1/ERK2 (data not shown). Thus, the shift toward stress (*e.g.*, p38 MAPK) and away from anti-apoptotic and cytoprotective (*e.g.*, AKT, ERK1/ERK2) signaling pathways, may contribute to the observed C75-induced synergistic potentiation of Taxol™ lethality under a concomitant schedule. On the contrary, pre-exposure to C75 strongly activates ERK1/ERK2 MAPK signaling, which, in turn, may raise the sensitivity threshold for Taxol™-induced cell death, then contributing to the observed lost of synergism between C75 and Taxol™ under a sequential schedule ["Fig. (**3**)"].

Although the FASN blocker C75 is not a DNA-damaging agent, it has been shown recently that FASN inhibitors can induce the accumulation of p53 and of its main down-stream effector, the inhibitor of cyclin-dependent kinases $p21^{WAF1/CIP1}$. Specifically, we have observed that pharmacological inhibition of FASN activity induces dose- and time-dependent accumulation of the CDKi $p21^{WAF1/CIP1}$ in both wild-type p53 cells (MCF-7) and mutant p53 cells (SK-Br3 and MDA-MB-231), although the degree of induction was greater in MCF-7 cells. These data suggest that the treatment with C75 in combination with Taxol™ may synergistically increase $p21^{WAF1/CIP1}$ protein levels, enhancing pro-apoptotic signals. It must be taken into account, however, that anti-mitotic drugs such as Taxol™ can induce mitotic arrest only in cells that enter mitosis; and in turn, prolonged mitotic arrest causes cell death. As cells are most sensitive against anti-mitotic drugs in M

phase, it is likely that C75, which produces inhibition of DNA replication and S phase progression preventing the tumor cells from entering the G_2-M phase of the cell cycle, would be antagonistic if C75 precedes anti-microtubule drugs exposure. In agreement with this speculation, sequential exposure to C75 followed by Taxol™ had strong antagonistic effects in MCF-7 cells. In this context, cytoprotective effects of p53-dependent $p21^{WAF1/CIP1}$ induction against paclitaxel-induced cytotoxicity have been demonstrated previously. This protection requires an intact $p53/p21^{WAF1/CIP1}$ pathway. In wild-type p53 MCF-7 cells, C75-induced p53-dependent $p21^{WAF1/CIP1}$ overexpression may cause complete cytoprotection by preventing entry into mitosis when cells are exposed to C75 first. Conversely, it has been demonstrated that lack of wild-type p53 or its transcriptional target, $p21^{WAF1/CIP1}$, allows cells to enter mitosis after DNA damage. Moreover, pretreatment with cytostatic doses of DNA-damaging drugs before treatment with anti-mitotic drugs results in selective cytotoxicity to cancer cells with defective $p53/p21^{WAF1/CIP1}$-dependent checkpoint. In mutant p53 and low-FAS-expressing MDA-MB-231 cells, sequential exposure to C75 followed by Taxol™ resulted in higher cytotoxicity than the simultaneous schedule. Therefore, although viewed as an unfavorable event, inactivation of p53 could be elegantly exploited for therapeutic advantage in designing clinical regimens including anti-metabolites directed against FASN. Accordingly, loss of p53 function substantially increases the sensitivity of tumor cells to FASN inhibitors in the absence of FASN overexpression.

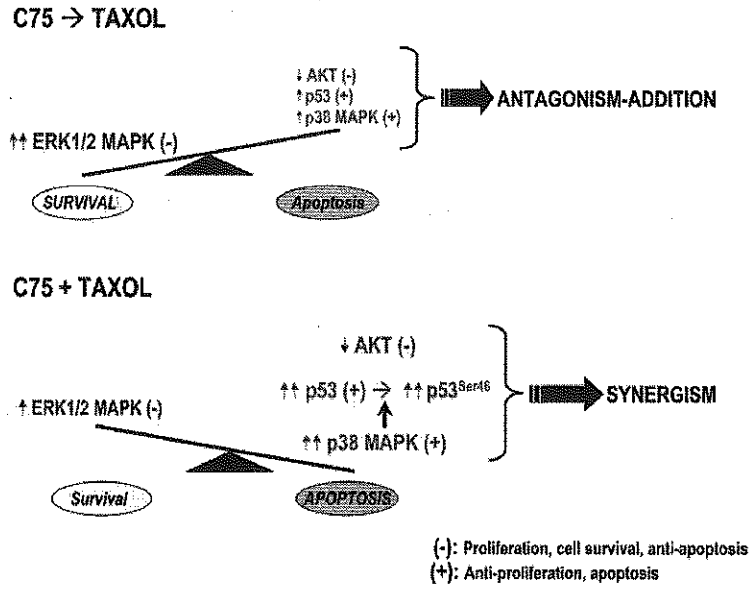


Fig. (3). Working model of signaling interactions in MCF-7 breast cancer cells exposed to FASN inhibitor C75 and Taxol™. In MCF-7 cells, exposure to C75 or Taxol™ leads to activation/inactivation of several signaling cascades involved in cell proliferation, survival and apoptosis. The relative outputs of these pathways may influence the apoptotic cell death response to these agents in a schedule-dependent manner. Exposure of C75-pretreated cells to Taxol™ does not oppose the sustained activation of the proliferation and cell survival MEK1/2 → ERK1/ERK2 MAPK pathway induced by C75 and Taxol™ as single agents, and modestly increases and decreases p38 MAPK and AKT activation, respectively. In contrast, the combined treatment of C75 and Taxol™ opposes the sustained ERK1/2 MAPK response to single treatments, markedly increases expression of phosphor-p38 MAPK which, in turn, phosphorylates p53 at Ser46, a pro-apoptotic post-transductional modification of p53, and significantly inactivates the anti-apoptotic AKT activity. Thus, the shift toward stress (*e.g.*, p38 MAPK, phosphor-$p53^{Ser46}$) and away from cytoprotective (*e.g.*, ERK1/2, AKT) signaling pathways, particularly when combined with as yet to be defined perturbations in cell cycle progression or mitotic events, may contribute to the observed C75-induced schedule-dependent potentiation of Taxol™ lethality in MCF-7 breast cancer cells.

FASN and Docetaxel (Taxotere™)

Docetaxel (Taxotere™) is a semi-synthetic paclitaxel belonging to the taxanes family. Similarly to paclitaxel, docetaxel acts as a mitotic spindle poison and induces mitotic block in proliferative cells. Docetaxel is effective as first-line treatment of metastatic breast cancer and is active in patients with paclitaxel-resistant breast cancer. Pre-clinical studies have demonstrated that breast cancer cells that overexpress the Her-2/*neu* oncogene are more resistant to docetaxel than those that do not overexpress Her-2/*neu*.

Mathematical assessment of the nature of cytotoxic interaction between FASN blockade and docetaxel. When we examined the relationship between breast cancer-associated FASN hyperactivity and HER-2/*neu*-induced breast cancer chemoresistance to the taxane docetaxel, we observed that co-administration of docetaxel and the mycotoxin cerulenin demonstrated strong synergism in HER-2/*neu*-overexpressing and docetaxel-resistant SK-Br3 cells, modest synergism in moderately HER-2/*neu*-expressing MCF-7 cells, and it showed additive effects in low HER-2/*neu*-expressing and docetaxel-sensitive MDA-MB-231 cells. Sequential exposure to cerulenin followed by docetaxel again yielded strong synergism in SK-Br3 cells, whereas antagonistic and moderate synergistic interactions were observed in MCF-7 and MDA-MB-231 cells, respectively.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and docetaxel. Although the ultimate mechanisms of action dictating that the synergistic effect of FASN blocker cerulenin *plus* docetaxel was limited only to SK-Br3 breast cancer cells were not fully addressed by our experiments, it can be suggested that down-regulation of *HER-2/neu* following cerulenin-induced inhibition of FASN activity forces *HER-2/neu*-overexpressing breast cancer cells that were originally resistant to docetaxel to become docetaxel sensitivity. Other investigators have demonstrated that *HER-2/neu* overexpression may be a dominant factor in conferring taxanes resistance on human breast cancer cells. Although once could argue that cerulenin-induced depletion of $p185^{HER\text{-}2/neu}$ in *HER-2/neu*-overexpressing breast cancer cells as assessed by ELISA may be unspecific, we recently demonstrated that inhibition of FASN activity by cerulenin or by the related synthetic FASN inhibitor C75 specifically down-regulates *HER-2/neu* oncogene expression at the protein level, as assessed by Western blotting, in *HER-2/neu* breast and ovarian cancer overexpressors. Moreover, FASN blockade in *HER-2/neu*-overexpressing breast cancer cells induced up-regulation of the transcriptional repressor of *HER-2/neu* PEA3, an *ets* DNA-binding protein that has been shown to inhibit *HER-2/neu*-promoted tumorigenesis by down-regulating *HER-2/neu* promoter activity, significantly decreased *HER-2/neu* gene transcription, and promoted a re-localization of *HER-2/neu* from the plasma membrane to the cytoplasmic/nuclear compartments.

FASN might be expressed and synthesized in coordination with increased demand for fatty acid metabolism and/or membrane synthesis in response to regulated or unregulated cell proliferation. Interestingly, the bulk of endogenously synthesized fatty acids are incorporated into membrane lipids by proliferating tumor cells. Therefore, inhibition of tumor-associated FAS activity may limit membrane synthesis required for cell growth and division, thus leading to non-specific chemosensitizing effects. However, pharmacological inhibition of FAS might result in changes in the lipid composition of tumor cell membrane. Accordingly, it has recently been shown that FAS plays a major role in the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains (raft-aggregates) in cancer cells. Raft-aggregates, which are defined as plasma membrane microdomains enriched with glycosphingolipids and cholesterol that render them insoluble in non-ionic detergents, are implicated in key cellular processes including signal transduction, intracellular trafficking, cell polarization, and cell migration. Many surface receptors are constitutively or inducibly associated with lipid rafts, and it has been suggested that the rafts function as platforms regulating the induction of signaling pathways. Remarkably, it has been described that clusters of *HER-2/neu* did colocalize with lipid rafts, and that the lipid environment did profoundly influence the association properties and biological function of *HER-2/neu* in SK-Br3 cells.

Although it can not be excluded that cerulenin and C75, in addition to the induction of *HER-2/neu* down-regulation, may also influence breast cancer cell chemosensitivity to docetaxel by other effects,

it is reasonable to suggest that *HER-2/neu* may play a pivotal role determining a specific enhancement of docetaxel toxicity in *HER-2/neu*-overexpressing breast cancer cells.

FASN and Vinorelbine (Navelbine™)

The *vinca* alkaloids represent one of the oldest classes of antineoplastic agents used in humans with a wide spectrum of activity against human tumors. Vinorelbine is a semi-synthetic analog that is known to inhibit microtubule polymerization. Clinical trials with single-agent vinorelbine or with vinorelbine incorporated into schedules using other agents have proven it to be effective against metastatic breast cancer.

Mathematical assessment of the nature of cytotoxic interactions between FASN blockade and vinorelbine. We examined the ability of the mycotoxin cerulenin, a potent and non-competitive inhibitor of FASN activity, to enhance the cytotoxic effects of vinorelbine (Navelbine™). SK-Br3, MCF-7 and MDA-MB-231 human breast cancer cell lines were employed as models of high, moderate and low levels of FASN ("cerulenin-target"), respectively. Combinations of cerulenin with vinorelbine were tested for synergism, additivity or antagonism using the isobologram and the median-effect plot (Chou-Talalay) analyses. Breast cancer cells were either simultaneously exposed to cerulenin and vinorelbine for 24 h or sequentially to cerulenin for 24 h followed by vinorelbine for 24 h. Concurrent exposure to cerulenin and vinorelbine resulted in synergistic interactions in MCF-7 and MDA-MB-231 cell lines, while additivity was found in SK-Br3 cells. Sequencing cerulenin followed by vinorelbine resulted in synergism for SK-Br3 and MDA-MB-231 cells, whereas it showed additive effects in MCF-7 cells. FASN activity blockade was found to synergistically enhance apoptosis-inducing activity of vinorelbine, as determined by an enzyme-linked immunosorbent assay for histone-associated DNA fragments.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and vinorelbine. Although our studies demonstrated that combinations of cerulenin with vinorelbine were mainly synergistic, the greatest number of synergistic combinations as well as the greatest magnitude of synergy was not observed in FASN-overexpressing SK-Br3 cells. Therefore, the presence of FASN overexpression by itself failed to correlate with the synergism between FASN inhibitor cerulenin and vinorelbine. Moreover, there was no correlation between intrinsic sensitivity to cerulenin and the occurrence of synergism, which was also evident in MDA-MB-231 cells expressing very low levels of FASN. We also observed that the synergism between cerulenin and vinorelbine was, in general, sequence-dependent. Thus, synergism was mainly observed when cells were exposed to the two agents simultaneously, whereas only additive or even antagonistic interactions were observed when cerulenin preceded vinorelbine. In MCF-7 cells, the cytotoxic interaction of cerulenin and vinorelbine was definitely schedule-dependent. These findings suggest that the simultaneous administration of FASN inhibitors and anti-mitotic drugs such as vinorelbine may be the optimal schedule for the combination in terms of cytotoxic effects.

Although the ultimate mechanisms of interaction operating with these schedules were not fully addressed by our experiments, analysis of the nature of the combination between two anti-neoplastic agents (synergism, addition, or antagonism) may provide some insight into the biochemical mechanisms of interactions of the drugs. For example, two drugs targeting the same enzyme or biochemical pathway may compete with one another resulting in an antagonistic interactions, whereas two drugs targeting completely independent pathways may be additive, and one drug which potentiates the action of another may result in therapeutic synergy. The anti-microtubule agent

vinorelbine is a promoter of apoptosis in cancer cells. Disruption microtubule structure by anti-mitotic agents results in induction of tumor suppressor gene p53 and the inhibitor of cyclin-dependent kinases, $p21^{WAF1/CIP1}$, which lead to apoptosis. Thus, it has been suggested that new pathways of anti-tumor agents could be directed at this p53 and $p21^{WAF1/CIP1}$ function, and may enhance the effect of existing agents (38). It has been reported that FASN inhibition produces rapid, profound inhibition of DNA replication and S phase progression in cancer cells, triggering apoptosis. It is thought that DNA damaged caused by radiation or various chemotherapeutic agents leads to an increase in the level of the tumor-suppressor protein p53, followed by a G_1 cell cycle arrest, sometimes accompanied by apoptosis. DNA-damage induced cell cycle arrest is also thought to be mediated by p53-dependent induction of the cyclin inhibitor protein $p21^{WAF1/CIP1}$. Although cerulenin is not a DNA-damaging agent, it has recently been shown that FAS inhibitors induced the accumulation of p53 and $p21^{WAF1/CIP1}$ proteins in RKO colon carcinoma cells. We have also observed that cerulenin induced dose- and time-dependent accumulation of the cyclin inhibitor $p21^{WAF1/CIP1}$ in both wild-type p53 cells (MCF-7) and mutant p53 cells (SK-Br3 and MDA-MB-231), although the degree of induction was greater in MCF-7 cells. Altogether, these data suggest that the treatment with cerulenin in combination with vinorelbine may synergistically increase $p21^{WAF1/CIP1}$ protein levels, enhancing pro-apoptotic signals. Accordingly, the inhibition of FASN activity with cerulenin together with vinorelbine resulted in an enhancement of apoptosis against wild-type p53 MCF-7 cells that was significantly higher than the additive value of the two drugs alone. Therefore, pharmacological inhibition of FASN activity synergistically increases the susceptibility of breast cancer cells to vinorelbine-induced apoptosis, a synergistic interaction that is equivalent to that obtained in cytotoxic studies using the isobologram and Chou-Talalay analyses. However, it must be taken into account that anti-mitotic drugs can induce mitotic arrest only in cells that enter mitosis; and in turn, prolonged mitotic arrest causes cell death. As cells are most sensitive against anti-mitotic drugs in M phase, it is likely that cerulenin, which produces inhibition of DNA replication and S phase progression preventing the tumor cells from entering the G_2-M phase of the cell cycle, would be antagonistic if cerulenin precedes anti-microtubule drugs exposure. In agreement with this speculation, sequential exposure to cerulenin followed by vinorelbine had additive or antagonistic effects in MCF-7 cells. In this context, cytoprotective effects of p53-dependent $p21^{WAF1/CIP1}$ induction against microtubule-active drugs have been demonstrated previously. This protection requires an intact $p53/p21^{WAF1/CIP1}$ pathway. In wild-type p53 MCF-7 cells, cerulenin-induced p53-dependent $p21^{WAF1/CIP1}$ overexpression may cause complete cytoprotection by preventing entry into mitosis when cells are exposed to cerulenin first. Conversely, it has been demonstrated that lack of wild-type p53 or its transcriptional target, $p21^{WAF1/CIP1}$, allows cells to enter mitosis after DNA damage. Moreover, pretreatment with cytostatic doses of DNA-damaging drugs before treatment with anti-mitotic drugs results in selective cytotoxicity to cancer cells with defective $p53/p21^{WAF1/CIP1}$-dependent checkpoint. In mutant p53 MDA-MB-231 and SK-Br3 cells, sequential exposure to cerulenin followed by vinorelbine resulted in higher cytotoxicity than the simultaneous schedule. Therefore, although viewed as an unfavorable event, inactivation of p53 could be elegantly exploited for therapeutic advantage in designing clinical regimens including anti-metabolites directed against FASN.

FASN and anti-metabolites

FASN and 5-Fluorouracil

The fluoropyrimidine 5-fluorouracil (5-FU) is an anticancer drug widely used in the treatment of human tumours. 5-FU was developed in the 1950s following the observation that rat hepatomas used the pyrimidine uracil more rapidly than normal tissues, indicating that uracil metabolism was a potential target for antimetabolite therapy. Antimetabolite drugs such as 5-FU work by inhibiting

essential biosynthetic processes, or by being incorporated into macromolecules, such as DNA and RNA, inhibiting their normal function. In this regard, the mechanism of cytotoxicity of 5-FU has been ascribed to the misincorporation of fluoronucleotides into RNA and DNA and to the inhibition of the nucleotide synthetic enzyme thymidilase synthase leading to DNA strands breaks and cell death. Over the past 20 years, increased understanding of the mechanism of action has led to the development of strategies aimed to increase the anticancer activity of 5-FU. Despite these advances, drug resistance remains a significant limitation to the clinical use of 5-FU.

Mathematical assessment of the nature of cytotoxic interactions between FASN blockade and 5-FU. Upon pharmacological inhibition of FASN activity using the natural antibiotic cerulenin, we evaluated the role of FASN-catalyzed endogenous fatty acid biogenesis on the sensitivity of SK-Br3, MCF-7 and MDA-MB-231 breast cancer cell lines to the anti-metabolite 5-FU. Cells were exposed simultaneously to cerulenin and 5-FU, sequentially to 5-FU followed by cerulenin or cerulenin followed by 5-FU. Cell viability was determined by MTT assays and the increase in 5-FU-induced cell growth inhibition was measured by dividing 5-FU IC_{30} and IC_{50} values (*i.e.*, 30% and 50% inhibitory concentrations, respectively) that were obtained in the absence of cerulenin by those in its presence. Co-exposure to cerulenin enhanced 5-FU efficacy up to 20-, 81-, and 58-times in SK-Br3, MCF-7 and MDA-MB-231 cells, respectively. Pre-treatment with cerulenin followed by the addition of 5-FU increased 5-FU efficacy up to 31-, 87-, and 126-times in SK-Br3, MCF-7 and MDA-MB-231 cells, respectively. Pre-treatment with 5-FU followed by the addition of cerulenin augmented 5-FU efficacy up to 107-, 20-, and 18-times in SK-Br3, MCF-7 and MDA-MB-231 cells, respectively. When isobologram transformations of multiple dose-response analyses were performed to detect *in vitro* synergy, we concluded that the nature of the interaction between cerulenin and 5-FU in individual breast cancer cells lines generally exhibited sequence-dependency. Thus, while synergism was mainly observed when breast cancer cells were exposed to 5-FU prior to cerulenin, moderate synergism or additive interactions was obtained either when the chemical FASN blocker preceded 5-FU or when both drugs were concurrently administered. Of note, no antagonist interactions occurred upon any schedule of combined treatment with cerulenin and 5-FU.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and 5-FU. Although the ultimate molecular mechanisms operating with these schedules are not addressed by our experiments it can be suggested various mechanisms by which FASN blockade leads to enhanced 5-FU efficacy in breast cancer cells:

a.) Cerulenin-induced FASN blockade inhibits phospholipids synthesis indirectly by reducing the intracellular pool of diacylglycerides, the direct precursors for phospholipids synthesis. Most of the cellular accumulation of phospholipids in cycling cells in preparation for mitosis takes place during the S-phase. In this sense, cells in S-phase are expected to be most sensitive to changes in phospholipids metabolism. Thus, a molecular mechanism underlying the synergism occurring when pre-treating cell with 5-FU prior to cerulenin might relate to the ability of 5-FU to promote accumulation of cells in early S-phase, making them more sensitive to the cytotoxic effects of cerulenin-induced FASN inhibition.

b.) Cancer cells have an unusual tolerance to limiting O_2 availability, with an atypical carbohydrate metabolism displaying high rates of anaerobic glycolysis which provides precursors for FAs and DNA synthesis. In addition, some studies have found that cancer cells exhibiting an increased resistance to chemotherapeutic agents are characterized by displaying a higher use of fat, cytosolic glycolysis and, when stressed, a reduced DNA damage. It has been also suggested that a novel

approach to enhance tumor c sensitivity to chemotherapy-induc cell death would be favourably tailor the cellular redox status. Interestingly, activation of FASN in cancer cells appears to represent a necessary survival strategy that occurs to compensate for an insufficiency of oxygen, providing oxidizing power for key oxidative steps, thus improving the redox balance despite the surrounding conditions of extreme hypoxia. Pharmacological inhibition of FASN activity may change the whole metabolic status of breast cancer cells, thus modulating their responses to extracellular stimuli perturbing redox balance, including the chemotherapeutic agent 5-FU.

c.) Upon cell entry the antimetabolite 5-FU is converted to its active form 5-fluoro-2´-deoxyuridine monophosphate (FdUMP). In the presence of a reduced folate cofactor FdUMP forms a stable complex with thymidilate synthase with resultant inhibition of DNA synthesis. In this regard, 5-FU can be viewed as a pure S phase-active chemotherapeutic agent. It has been reported that FASN inhibitors produce rapid, profound inhibition of DNA replication and S phase progression in human cancer cells (23). Moreover, it has been recently reported that cerulenin-mediated apoptosis is involved in adenine metabolic biosynthetic pathway. Thus, pre-treatment of cells with the FASN inhibitor cerulenin would lead to DNA synthesis inhibition in cancer cells, blocking 5-FU incorporation to DNA during replication and therefore making immediate 5-FU treatment less effective.

d.) It has been reported that certain specific proteins involved in glycolysis, FA synthesis (including FASN) and detoxification pathways are more highly expressed in HER2/*neu*-positive breast tumours when compared with HER2/*neu*-negative breast tumours. In addition, HER2/*neu* overexpression is associated with an increased resistance to multiple chemotherapeutic agents including MIAs, cisplatin or 5-FU. Considering our earlier reports revealing that FASN inhibition specifically suppresses HER2/*neu* overexpression in cancer cells, the synergistic interactions occurring between 5-FU and cerulenin in FASN- and HER2/*neu*-overexpressing breast cancer cells can be explained, at least in part, through FASN inhibition-related depletion of HER2/*neu*. In this sense, our current results support the notion that HER2/*neu* oncogene may act as the key molecular sensor of metabolism imbalance after the perturbation of tumor-associated FASN hyperactivity in breast cancer cells.

FASN and HER network-targeted therapies

FASN and trastuzumab

Trastuzumab (Herceptin®) is a recombinant anti-Her-2/*neu* monoclonal antibody directed at the $p185^{Her\text{-}2/neu}$ ectodomain that is active against tumor cells that overexpress Her-2/*neu* oncogene. However, not all Her-2/*neu*-overexpressing cancer cells respond to treatment with trastuzumab. Moreover, its clinical benefit is limited by the fact that most breast cancers become resistant to trastuzumab in less than 12 months.

Mathematical assessment of the nature of cytotoxic interactions between FASN blockade and trastuzumab. We explored whether the molecular connection between FASN and HER-2/*neu* could be simultaneously targeted therapeutically. For this purpose, we analyzed the cell growth inhibitory interactions between cerulenin and the humanized anti-HER-2/*neu* antibody trastuzumab. The IC_{30} values of trastuzumab were measured for HER-2/*neu*-overexpressing SK-Br3 and BT-474 cells after a 72 h treatment in the presence or absence of a given concentration of cerulenin, and the degree of potentiation of trastuzumab efficacy was expressed as a sensitization factor by dividing the IC_{30}

values in the absence of cerulenin by those in the presence of cerulenin. From our data we concluded that cerulenin-induced blockade of FASN activity dramatically enhanced the growth-inhibitory activity of trastuzumab in a dose-dependent manner. The most significant changes were seen in SK-Br3 cells, in which a co-exposure to 1.5 μg/ml cerulenin sensitized cells to trastuzumab by 200-fold. For BT-474 cells, cerulenin co-exposure increased trastuzumab efficacy up to 52-fold. Similarly, the degree of potentiation of cerulenin efficacy by trastuzumab co-exposure was evaluated by dividing the IC_{30} values of cerulenin the absence of trastuzumab by those in the presence of trastuzumab. In SK-Br3 cells a 72 h co-exposure to 20 μg/ml trastuzumab sensitized cells to cerulenin by 8-fold. For BT-474 cells, 20 μg/ml trastuzumab co-exposure significantly increased cerulenin efficacy by 38-fold. Because it was clearly apparent that this sensitization was due, at least in part, to the cell toxicity of both drugs themselves, the precise nature of the interaction between cerulenin and trastuzumab was investigated using the isobologram analysis. When the experimental isoeffect points (the concentrations of cerulenin and trastuzumab which combined produced 30% reduction in survival of SK-Br3 and BT-474 cells) were plotted and compared to the additive line, the data points fell on the left side of the line, suggesting a supra-additive or synergistic interaction of the two agents. When Student's *t* tests were computed to evaluate whether significant differences in the combination index (CI) values occurred as compared to a null hypothesized CI of 1 (additivity), and to formally evaluate whether synergism was evident, the average sum of the fractional effects in SK-Br3 cells was 0.562 ± 0.197 ($p = 0.001$), indicating that the amount of the two drugs together necessary to reduce SK-Br3 cell viability by 30% was only 0.562 times as much as would be required if they demonstrated purely additive behavior. In BT-474 cells, the mean sum of the fractional effects was 0.662 ± 0.266 ($p = 0.007$).

Trastuzumab at a dose of 20 μg/ml caused less than 15% reduction in the cell viability of moderately HER-2/*neu*-expressing T47-D and low HER-2/*neu*-expressing MCF-7 breast cancer cells. We selected this dose to study whether any cooperative anti-proliferative effect may occur between cerulenin and trastuzumab in not HER-2/*neu*-overexpressing breast cancer cells. When increasing concentrations of cerulenin were used in combination with trastuzumab in the moderately HER-2/*neu*-expressing T47-D cells, we found that the antiproliferative effect was clearly cooperative or supra-additive. 3 μg/ml cerulenin and 20 μg/ml trastuzumab, which used alone caused 36% and 5% cytotoxicity, respectively, showed a 62% decrease in the cell viability. An additive growth-inhibitory effect was observed in HER-2/*neu*-negative MCF-7 cells co-treated with trastuzumab and increasing concentrations of cerulenin. Taken together, these results demonstrate that a synergistic augmentation of the trastuzumab efficacy by cerulenin specifically takes place in HER-2/*neu*-overexpressing and moderately HER-2/*neu*-expressing breast cancer cells.

The synergism between cerulenin and trastuzumab was sequence-dependent. Thus, a synergistic interaction took place when HER-2/*neu*-positive cells were exposed to the two agents simultaneously, whereas only additive or even antagonistic interactions were observed when cerulenin preceded trastuzumab or when the cells were exposed to trastuzumab prior to cerulenin. A similar picture emerged when the β-lactone orlistat was combined with trastuzumab. Our studies demonstrated that the greatest number of synergistic combinations as well as the greatest magnitude of synergy was observed when HER2/*neu*- and FASN-overexpressing SK-Br3 breast cancer cells were exposed to the two agents simultaneously, whereas additive or even antagonistic interactions were observed when either FASN blocker orlistat preceded trastuzumab or trastuzumab preceded orlistat, respectively. When the combination of Orlistat and trastuzumab in either concurrent (Orlistat + trastuzumab) or sequential (Orlistat → trastuzumab; trastuzumab → Orlistat) schedules was tested for synergism, addition or antagonism using the combination index (CI) method of Chou-Talalay, co-exposure of Orlistat and trastuzumab demonstrated strong synergistic effects ($CI_{10\text{-}90} = 0.110\text{-}0.847$), whereas sequential exposure to Orlistat followed by trastuzumab ($CI_{10\text{-}90} = 0.380\text{-}1.210$) and

trastuzumab followed by Orlistat (CI_{10-90} = 0.605-1.278) mainly showed additive or antagonistic interactions. These findings suggest that co-exposure of Her2/*neu*-overexpressing SK-Br3 cells to FASN inhibitor orlistat and anti-Her2/*neu* antibody trastuzumab is necessary for maximal augmentation of cytotoxicity, whereas sequential administration of trastuzumab followed by orlistat significantly reduces the synergism between the two agents.

To determine whether the synergy between cerulenin and trastuzumab was accompanied with an increase in the extent of apoptosis, a combination of both agents was used to treat SK-Br3 cells, and cell death was first measured by the Cell Death Detection ELISA, which is based on quantitative enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes, which are released into the cytoplasm of cells dying from apoptosis. The *x*-fold increase in apoptosis was calculated by comparing the optical density readings from cerulenin-, trastuzumab-, and cerulenin *plus* trastuzumab-treated samples with the values of the untreated controls. The combination of cerulenin *plus* trastuzumab synergistically enhanced the basal level of cell death. In SK-Br3 cells, 2 μg/ml cerulenin and 10 μg/ml trastuzumab combined caused five times more cell death than trastuzumab alone, and twice more cell death than cerulenin alone. Similarly, SK-Br3 cells co-treated with trastuzumab and orlistat exhibited a higher degree of cell death compared with that observed when trastuzumab and orlistat were used as single agents. Therefore, the increased sensitivity to trastuzumab seen in orlistat-treated SK-Br3 cells is not simply the result of changes in cell proliferation, but might actually be due to increases in apoptotic cell death following orlistat-induced cell damage.

To assess if the cell death observed above represented apoptosis, and also to assess the minimal concentration of cerulenin, which when combined with trastuzumab caused supra-additive cell death, we next focused on an apoptotic effect of the combination of cerulenin and trastuzumab in SK-Br3 cells as measured by the enzymatic *in situ* labeling of apoptosis-induced DNA double-strand breaks involving DNA polymerase and Terminal deoxynucleotidyl Transferase (TdT) tailing reactions (TUNEL assay). Individually, 1 μg/ml cerulenin and 5 μg/ml trastuzumab caused slight apoptosis (13% and 2%, respectively) as measured by the number of TUNEL-positive cells. When cells were simultaneously treated with both drugs, there was a significant increase in the number of apoptotic TUNEL-positive cells to 28%. These results establish that the combined treatment with cerulenin and trastuzumab synergistically enhances apoptosis in HER-2/*neu*-overexpressing breast cancer cells.

Molecular mechanisms underlying the cytotoxic interactions between FASN blockade and trastuzumab. Transcriptional repression of Her2/*neu* gene occurring upon pharmacological blockade of FASN activity, when concurrently combined with sub-optimal concentrations of trastuzumab, appears to promote high levels of apoptotic cell death in Her2/*neu*-overexpressing breast cancer models. Transfection of HER-2/*neu* gene into MDA-MB-231 cells dramatically increased cerulenin efficacy in the absence of FASN protein levels comparable to those found in FASN-overexpressing SK-Br3 cells. Thus, a greater expression of HER-2/*neu* gene product seems to determine a greater sensitivity to cytotoxicity that follows inhibition of FASN activity, with HER-2/*neu* overexpression predicting synergy between chemical inhibitors of FASN and trastuzumab. More importantly, the combination of FASN inhibitors and trastuzumab was more effective than the single treatments in inhibiting the expression of HER-2/*neu* oncogene, and inducing apoptosis.

Although the ultimate molecular mechanisms of interaction operating with these schedules were not conclusively addressed by our experiments, these events may be related to the relative output of Her2/*neu*-driven signaling in breast cancer cells. Moreover, these findings support the clinical potential of concurrent treatments using FASN blockers, which seem to repress Her2/*neu* oncogene expression at the transcriptional levels by up-regulating PEA3, and monoclonal antibodies to Her2/*neu* such as trastuzumab, which targets the ectodomain of $p185^{Her2/neu}$ and promotes Her2/*neu* protein degradation. Pre-exposure to orlistat does not affect FASN expression, while significantly down-regulating Her2/*neu* expression. This, in turn, may raise the sensitivity threshold for trastuzumab-induced cell growth inhibition, then contributing to the observed reduction of synergism under a sequential schedule orlistat → trastuzumab. Pre-exposure to trastuzumab not only reduces $p185^{Her2/neu}$ but further down-regulates FASN. Under these conditions, trastuzumab pretreatment not only decreases the levels of orlistat's target (*i.e.* FASN) but further blocks one of the molecular mechanisms through which FASN blockade promotes breast cancer cell toxicity (*i.e.* Her2/*neu* down-regulation). Accordingly, the trastuzumab → orlistat schedule yields the highest CI (*i.e.* antagonistic) values in all the combinations tested for orlistat and trastuzumab. Kumar-Sinha *et al.* observed that cell toxicity mediated by the FASN inhibitor C75 is blocked upon pre-treatment of HER-2/*neu*-overexpressing cells with CI-1033, an irreversible inhibitor for *HER* kinases. From a clinical perspective, these findings suggest that the simultaneous administration of orlistat-related anti-FAS compounds and trastuzumab may be the optimal schedule for the combination in terms of cytotoxic effects.

Furthermore, because HER-2/*neu* overexpression induces chemoresistance to certain chemotherapeutic agents such as Taxol, it has been suggested that PEA3-induced down-regulation of HER-2/*neu* could enhance the therapeutic efficacy of Taxol or other anticancer drugs. Interestingly, cerulenin co-exposure synergistically enhances the cytotoxic effects of microtubule-interfering agents (*i.e.* paclitaxel, docetaxel, and vinorelbine) in HER-2/*neu*- and FASN-overexpressing SK-Br3 cells.

Remarks, conclusions and perspectives

Studies with chemical inhibitors and, more recently, with RNAi-mediated down-regulation of FASN expression have revealed that FASN inhibition has a dramatic impact on cancer cells. Cancer cells stop proliferating and ultimately die, at least in part, through the process of apoptosis. Therefore, FASN and the endogenous fatty acid synthesis pathway provide a number of avenues of future exploration not only applicable to the diagnosis and prognosis of human cancer but further to its prevention and treatment.

The homodimeric mammalian FASN is one of the most complex mutienzymes, in that each 250/270-kD polypeptide chain carries all seven functional domains required for fatty acid synthesis, and in which substrates are handed from one functional domain to the next. The combination of FASN structural complexity and until recently the lack of X-ray crystallography data of mammalian FASN created a significant challenge in the exploitation of FASN as a valuable target for drug development. Comparison of the FASN monomer and dimer structures suggests that the FASN monomers in the dimeric form of the enzyme are not aligned side by side in a fully extended, antiparallel fashion, as proposed in the conventional model for FASN organization. Indeed, as recently demonstrated, the FASN monomers adopt a coiled conformation allowing for a variety of intra- and intermonomer functional domain interactions, with the KS domains located in the central position of the FASN structure. It is hoped that this latest 4.5 Å resolution X-ray crystallographic map of mammalian FASN will lay a basis for efforts at structure-based drug design with this target. While we expect that the improvement in the selectivity and potency of forthcoming novel FASN-targeted

small molecule inhibitors will direct the foundation of a new family of chemotherapeutic agents in cancer history, several reports from our group describing how FASN-catalyzed endogenous fatty acid biogenesis also participates on the response of cancer cells to chemo- and immuno-therapies, definitely support the notion of FASN as an attractive target for chemoprevention or curative treatment for nearly all human epithelial malignancies.

Key research accomplishments

- Chemical FASN blockers as well as RNA silencing interference techniques directed against *FASN* gene may provide a mean to increase efficacy over existing therapy in human breast carcinomas.
- FASN-related "lipogenic phenotype" actively regulates the efficacy of microtubule-interfering agents (*i.e.*, the taxanes paclitaxel and docetaxel and the vinca alkaloid vinorelbine), anthracyclines (*i.e.*, doxorubicin), nucleotide analogues (*i.e.*, 5-Fluorouracil), and monoclonal antibodies (*i.e.*, trastuzumab) in cultured breast cancer cells.
- Inherent and acquired drug resistance hampers successful treatment in many human malignancies, and its prevention or reversal is still awaiting new therapeutic strategies or pharmaceuticals. While current research is focused on producing the ideal FASN blocker and it is hoped that the latest 4.5 Å resolution X-ray crystallographic map of mammalian FASN will lay a basis for efforts at structure-based drug design with this target, we present evidence to suggest that FASN-catalyzed endogenous FA biogenesis is a previously unrecognized respondent mechanism to drug-induced cell injuries.
- The fact that breast cancer cells differing in FASN levels significantly differ as well in their sensitivity to currently used drug regimens should impose new challenges for the future use of anti-FASN strategies in the chemoprevention or curative treatment of breast cancer disease.

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Arch Immunol Ther Exp, 2004, 52, 414–426
PL ISSN 0004-069X

WWW.AITE-ONLINE.ORG
Review

Received: 2004.08.04
Accepted: 2004.09.21
Published: 2004.12.15

Fatty acid synthase-catalyzed *de novo* fatty acid biosynthesis: from anabolic-energy-storage pathway in normal tissues to jack-of-all-trades in cancer cells

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Source of support: Javier A. Menendez is the recipient of a Translational Research Pilot Project (PP2) from the Specialized Program of Research Excellence (SPORE) in Breast Cancer (Robert H. Lurie Comprehensive Cancer Center, Chicago, USA), of a Basic, Clinical and Translational Award (BRCTR0403141) from the Susan G. Komen Breast Cancer Foundation (USA), and of a Breast Cancer Concept Award (BC033538) from the Department of Defense (DOD, USA).

Summary

In 1994, Kuhajda and colleagues unambiguously identified the oncogenic antigen-519, a prognostic molecule found in breast cancer patients with markedly worsened prognosis, as fatty acid synthase (FAS), the key enzyme for the *de novo* fatty acid biosynthesis. It now appears that human carcinomas and their pre-neoplastic lesions constitutively overexpress FAS and undergo significant endogenous fatty acid biosynthesis. Moreover, FAS blockade specifically induces apoptotic cancer cell death and prolongs survival of cancer xenograft hosts. Therefore, FAS signaling seems to play a central role in the maintenance of the malignant phenotype by enhancing cancer cell survival and proliferation. This review documents the rapidly changing perspectives on the function of FAS in cancer biology. First, we describe molecular mechanism by which aberrant transduction cascades driven by oncogenic changes subvert the down-regulatory effects of dietary fatty acids, resulting in tumor-associated FAS insensitivity to nutritional signals. Second, we speculate on the putative function that hypoxia can play as the epigenetic factor that triggers and maintains FAS overexpression in cancer cells by inducing changes in gene expression and in metabolism for survival. Third, we explore the role that FAS exhibits in cancer evolution by specifically regulating cancer-related proteins such as Her-2/*neu* oncogene and estrogen receptor. Finally, we reveal previously unrecognized functions of FAS on the response of cancer cells to chemo-, endocrine-, and immuno-therapies. These findings, all together, should ultimately enhance our understanding of how FAS-dependent endogenous fatty acid metabolism, once considered a minor anabolic-energy-storage pathway in normal cells, has become a jack-of-all-trades in cancer cells.

Key words: **fatty acid synthase • fatty acids • oncogene • cancer • metabolism**

Full-text PDF: http://www.alte-online/pdf/vol_52/no_6/6563.pdf

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Drug News Perspect 18(6), July/August 2005

Available on the web at: www.prous.com/journals

LOOKING AHEAD

FAS-dependent endogenous fatty acid metabolism may represent a valuable molecular avenue for developing FAS-based chemopreventive and/or chemotherapeutic strategies in Her-2/neu–related breast cancer.

Targeting Fatty Acid Synthase: Potential for Therapeutic Intervention in Her-2/*neu*–Overexpressing Breast Cancer

by Javier A. Menendez, Ruth Lupu and Ramon Colomer

Summary

Fatty acid synthase (FAS)-catalyzed *de novo* fatty acid biosynthesis, an anabolic energy-storage pathway largely considered of minor importance in humans, actively contributes to the cancer phenotype by virtue of its ability to specifically regulate the expression and activity of Her-2/*neu* (*erb*B-2) oncogene. First, a positive correlation between high levels of FAS expression and/or activity and the amplification and/or overexpression of Her-2/*neu* oncogene exists in human breast cancer cell lines. Second, Her-2/*neu* overexpression stimulates the activity of FAS gene promoter and ultimately mediates increased endogenous fatty acid biosynthesis, while this Her-2/*neu*-induced upregulation of breast cancer-associated FAS is inhibitable by anti-Her-2/*neu* antibodies such as trastuzumab (*Herceptin*™). Third, pharmacological inhibition of FAS activity negatively regulates the expression and tyrosine-kinase activity of Her-2/*neu*-coded $p185^{Her\text{-}2/neu}$ oncoprotein.

One of the most frequent phenotypic alterations in cancer cells is the overexpression and hyperactivity of fatty acid synthase (FAS), the key enzyme responsible for the *de novo* synthesis of long-chain fatty acids through catalyzing the NADPH-dependent condensation of acetyl-CoA and malonyl-CoA.[1–5] The early and nearly universal upregulation of FAS in most human cancers and the association of FAS overexpression with poor clinical outcome both reveal a necessary role of FAS in the development, maintenance and/or enhancement of the malignant cancer phenotype.[1–3] However, the increased expression of FAS gene and of FAS-catalyzed endogenous fatty acid biogenesis in tumor cells appears to be part of a more general change in the genetic program controlling lipogenesis, as evidenced by the concomitant increase of other enzymes of the same lipogenic pathway.[6,7] Therefore, one of the main questions arising from the above studies is whether activation of FAS in neoplastic cells is a mere manifestation of an early and common deregulation of upstream regulatory pathways or contributes actively to the cancer phenotype. Indeed, most of the current notions plead for an epigenetic basis of increased FAS expression in cancer cells and suggest that changes in upstream regulatory circuits (e.g., hormones–hormone receptors, growth factors–growth factor receptors, lipogenic transcription

COMMENTS

Orlistat: From Antiobesity Drug to Anticancer Agent in Her-2/*neu* (*erb*B-2)-Overexpressing Gastrointestinal Tumors?

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The U.S. Food and Drug Administration (FDA)-approved antiobesity drug Orlistat (marketed by Roche [Basel, Switzerland] as Xenical) is a semisynthetic derivative of lipstatin that irreversibly inhibits pancreatic and gastric lipases within the gastrointestinal (GI) tract (1). Interestingly, an intense public interest has been generated by the characterization of Orlistat as a potent inhibitor of prostate and breast tumor growth (2). Mechanistically, it has been demonstrated that Orlistat exhibits antiproliferative and antitumor properties toward prostate and breast cancer cells by virtue of its ability to block the lipogenic activity of fatty acid synthase (FAS) (2, 3). Fatty acid synthase is the key metabolic muti-enzyme that is responsible for the terminal catalytic step in the *de novo* fatty acid biosynthesis (4). Upregulation of FAS expression and activity, an anabolic-energy-storage pathway largely considered of minor importance in humans, is a very early and nearly universal neoplastic marker that positively correlates with aggressive behaviors and poorer clinical outcome prognoses in many human cancers (5). Interestingly, we recently demonstrated that cancer-associated FAS, which to date appeared to be part of a growth factor-driven pleiotropic change in the genetic program controlling lipogenesis, actively contributes to the cancer phenotype by regulating the expression, activity, and cellular localization of the Her-2/*neu* (*erb*B-2) oncogene (6), a master player in the etiology and aggressive behavior of several cancers (7).

Because Orlistat possesses extremely low oral bioavailability, a novel formulation will be required for treating tumors such as prostate or breast carcinomas. However, we hypothesized that Orlistat-induced inhibition of FAS activity may represent a valuable therapeutic strategy to test in GI tumors overexpressing Her-2/*neu* oncogene, that is, associated with approximately one fourth of all GI tract malignancies (8). To test this hypothesis, we employed the highly metastatic and Her-2/*neu*-overexpressing NCI-N87 stomach carcinoma cell line. Micromolar concentrations of Orlistat (up to 40 μM) induced dose-dependent antiproliferative effects against NCI-N87 cells when relative cell numbers were determined using a 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT)-based characterization of metabolically viable cells (Fig. 1a). Cell cycle analyses revealed that Orlistat exposure induced a complete loss of G_2-M cell population with a concomitant increase of cells in G_1 by 48 hrs. This G_1 blockade of Orlistat-treated NCI-N87 cells was accompanied with significant increases

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1535-3702/05/2303-0151$15.00

Medical Hypotheses (2004) x, xxx–xxx

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Targeting fatty acid synthase-driven lipid rafts: a novel strategy to overcome trastuzumab resistance in breast cancer cells

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Received 16 September 2004; accepted 18 September 2004

Summary Trastuzumab (Herceptin™) is a humanized antibody directed against the extracellular domain of the tyrosine kinase orphan receptor Her-2/*neu* (*erb*B-2) that has shown therapeutic efficacy against Her-2/*neu*-overexpressing breast tumors. However, less than 35% of patients with Her-2/*neu*-overexpressing metastatic breast cancer respond to trastuzumab as a single agent, whereas the remaining cases do not demonstrate tumor regression. Furthermore, the majority of patients who achieve an initial response generally acquire resistance within one year. Therefore, the identification of the potential mechanisms of resistance to trastuzumab can be very helpful for the development of new compounds, which might overcome that resistance and/or have additive/synergistic antitumor effect when given in association with trastuzumab. Recent studies in breast cancer cells have revealed a bi-directional connection between Her-2/*neu* and fatty acid synthase (FAS), a major lipogenic enzyme catalyzing the synthesis of long-chain saturated fatty acids from the 2-carbon donors malonyl-CoA and acetyl-CoA. Her-2/*neu* overexpression stimulates the FAS promoter and ultimately mediates increased endogenous fatty synthesis, and this Her-2/*neu*-mediated induction of breast cancer-associated FAS is inhibitable by trastuzumab. On the other hand, chemical FAS inhibitors as well as RNA interference-mediated silencing of FAS gene repress Her-2/*neu* gene expression at the transcriptional level. Moreover, specific FAS blockade synergistically sensitizes breast cancer cells carrying Her-2/*neu*-oncogene amplification and/or overexpression to trastuzumab-induced cell growth inhibition and apoptotic cell death. Strikingly, FAS inhibition synergistically interacts with trastuzumab in Her-2/*neu*-negative breast cancer cells engineered to overexpress Her-2/*neu*, thus suggesting that the molecular linkage between FAS activity and functioning of Her-2/*neu* cannot be explained only on the basis of a transcriptional repression of Her-2/*neu* gene promoter. Interestingly, while in liver and adipose tissue FAS produces fat from excess carbon consumed as carbohydrates, which is ultimately stored as triglycerides, in epithelial cancer cells, FAS activity is mainly involved in the production of phospholipids partitioning into detergent-resistant membrane microdomains (lipid raft-aggregates), which point to an active role of FAS in the deregulation of membrane functioning in tumor cells. Importantly, clusters of Her-2/*neu* and EGFR (*erb*B-1) co-localize with lipid rafts and the lipid environment in the cell membrane of breast cancer cells

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0306-9877/$ - see front matter
doi:10.1016/j.mehy.2004.09.027

Effect of γ-Linolenic Acid on the Transcriptional Activity of the Her-2/neu (erbB-2) Oncogene

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The ω-6 polyunsaturated fatty acid γ-linolenic acid (GLA; 18:3n-6), which is found in several plant oils and is used as an herbal medicine, has antitumor activity in vitro. We examined the effect of GLA on the expression of the Her-2/neu (erbB-2) oncogene, which is involved in development of numerous types of human cancer. Flow cytometric and immunoblotting analyses demonstrated that GLA treatment substantially reduced Her-2/neu protein levels in the Her-2/neu–overexpressing cell lines BT-474, SK-Br3, and MDA-MB-453 (breast cancer), SK-OV3 (ovarian cancer), and NCI-N87 (gastrointestinal tumor derived). GLA exposure led to a dramatic decrease in Her-2/neu promoter activity and a concomitant increase in the levels of polyomavirus enhancer activator 3 (PEA3), a transcriptional repressor of Her-2/neu, in these cell lines. In transient transfection experiments, a Her-2/neu promoter bearing a PEA3 site–mutated sequence was not subject to negative regulation by GLA in Her-2/neu–overexpressing cell lines. Concurrent treatments of Her-2/neu–overexpressing cancer cells with GLA and the anti–Her-2/neu antibody trastuzumab led to synergistic increases in apoptosis and reduced growth and colony formation. [J Natl Cancer Inst 2005;97:1611–5]

The oil from seeds of the evening primrose is used in traditional medicine as a treatment for a variety of chronic diseases. This oil (and that from seeds of borage and black currant) contains γ-linolenic acid (GLA), a member of the ω-6 family of polyunsaturated fatty acids. GLA exerts selective cytotoxic effects on cancer cells without affecting normal cells *(1–5)*. In addition, exogenous supplementation with GLA sensitizes breast cancer cells to antimitotic drugs and endocrine therapies *(6–9)*. A recent phase II study suggested that GLA may have activity against endocrine-sensitive breast cancer with low systemic toxicity *(10)*, and GLA treatments have led to some tumor responses in other advanced solid malignancies *(11–15)*. Although enhanced lipid peroxidation has been proposed as the main mechanism of action of GLA *(1–5)*, the ultimate molecular pathways underlying GLA's anticancer actions remain largely unknown.

A novel molecular explanation concerning the anticancer actions of GLA may relate to its ability to specifically regulate oncoproteins. We recently reported that exogenous supplementation of cultured breast cancer cells with GLA significantly diminished proteolytic cleavage of the extracellular domain of the Her-2/neu–coded $p185^{Her\text{-}2/neu}$ tyrosine kinase oncoprotein and, consequently, its activation *(16)*. Considering that activation and overexpression of Her-2/neu oncogene are crucial for the etiology, progression, and cell sensitivity to various treatments in ~30% of breast carcinomas *(17–32)*, these findings showed a previously unrecognized mechanism by which GLA might regulate breast cancer cell growth, metastasis formation, and response to chemotherapy and endocrine therapy. However, two main questions remained to be addressed: (1) Does GLA-induced deactivation of $p185^{Her\text{-}2/neu}$ relate to GLA-induced changes in Her-2/neu gene expression? (2) Is the ability of GLA to regulate Her-2/neu oncogene a common mechanism of GLA's action against other types of cancer or is it restricted to breast cancer?

To characterize the effects of GLA on the expression of Her-2/neu oncogene, we first treated BT-474 and SK-Br3 breast cancer cells, which naturally contain Her-2/neu oncogene amplification *(33,34)*, with GLA (10 μg/mL for 48 hours). In flow cytometry analyses, levels of cell surface–associated Her-2/neu protein were substantially lower in GLA-treated cells than in vehicle-treated cells (Fig. 1, A). Similarly, immunoblot analysis indicated that GLA treatment led to a substantial reduction in Her-2/neu protein levels in both cell lines (Fig. 1, B).

Although Her-2/neu overexpression was originally attributed solely to erbB-2 gene amplification, an elevation in Her-2/neu mRNA levels per gene copy is also observed in all cell lines that exhibit gene amplification *(35)*. We used reporter gene expression and reverse transcription–polymerase chain reaction (RT-PCR) analyses to characterize the effects of GLA on the transcription of the Her-2/neu gene. Treatment of BT-474 and SK-Br3 cells that had been transfected with a construct containing a luciferase reporter gene driven by a wild-type Her-2/neu promoter fragment with GLA (10 μg/mL for 48 hours) led to a strong reduction in reporter gene expression in both lines (Table 1).

For semiquantitative RT-PCR analyses, we treated BT-474 and SK-Br3 cells with varying concentrations of GLA (5, 10, or 20 μg/ml for 48 hours) and then extracted total RNA from the cells. One microgram of total RNA was then reversed transcribed and amplified with specific primers for Her-2/neu, and the products were separated on agarose gels (Fig. 1, C). We observed strong, dose-dependent decreases in transcription of the Her-2/neu gene in both cells lines with GLA treatment.

We next investigated the possibility that GLA-induced repression of Her-2/neu

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See "Notes" following "References."

DOI: 10.1093/jnci/dji343

Int. J. Cancer: **115**, 19–35 (2005)

FAST TRACK

Pharmacological and small interference RNA-mediated inhibition of breast cancer-associated fatty acid synthase (oncogenic antigen-519) synergistically enhances Taxol (Paclitaxel)-induced cytotoxicity

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The relationship between breast cancer-associated fatty acid synthase (FAS; oncogenic antigen-519) and chemotherapy-induced cell damage has not been studied. We examined the ability of C75, a synthetic slow-binding inhibitor of FAS activity, to modulate the cytotoxic activity of the microtubule-interfering agent Taxol™ (paclitaxel) in SK-Br3, MDA-MB-231, MCF-7 and multidrug-resistant MDR-1 (P-Glycoprotein)-overexpressing MCF-7/AdrR breast cancer cells. When the combination of C75 with Taxol™ in either concurrent (C75 + Taxol™ 24 hr) or sequential (C75 24 hr → Taxol™ 24 hr) schedules were tested for synergism, addition or antagonism using the isobologram and the median-effect plot analyses, co-exposure of C75 and Taxol™ mostly demonstrated synergistic effects, whereas sequential exposure to C75 followed by Taxol™ mainly showed additive or antagonistic interactions. Because the nature of the cytotoxic interactions was definitely schedule-dependent in MCF-7 cells, we next evaluated the effects of C75 on Taxol™-induced apoptosis as well as Taxol™-activated cell death and cell survival-signaling pathways in this breast cancer cell model. An ELISA for histone-associated DNA fragments demonstrated that C75 and Taxol™ co-exposure caused a synergistic enhancement of apoptotic cell death, whereas C75 pre-treatment did not enhance the apoptosis-inducing activity of Taxol™. Co-exposure to C75 and Taxol™ induced a remarkable nuclear accumulation of activated p38 mitogen-activated protein kinase (p38 MAPK), which was accompanied by a synergistic nuclear accumulation of the p53 tumor-suppressor protein that was phosphorylated at Ser46, a p38 MAPK-regulated pro-apoptotic modification of p53. As single agents, FAS blocker C75 and Taxol™ induced a significant stimulation of the proliferation and cell survival mitogen-activated protein kinase extracellular signal-regulated kinase (ERK1/ERK2 MAPK) activity, whereas, in combination, they interfered with ERK1/ERK2 activation. Moreover, the combined treatment of C75 and Taxol™ inactivated the anti-apoptotic AKT (protein kinase B) kinase more than either agent alone, as evidenced by a synergistic down-regulation of AKT phosphorylation at its activating site Ser^{473} without affecting AKT protein levels. To rule out a role for non-FAS C75-mediated effects, we finally used the potent and highly sequence-specific mechanism of RNA interference (RNAi) to block FAS-dependent signaling. Importantly, SK-Br3 and multi-drug resistant MCF-7/AdrR cells transiently transfected with sequence-specific double-stranded RNA oligonucleotides targeting FAS gene demonstrated hypersensitivity to Taxol™-induced apoptotic cell death. Our findings establish for the first time that FAS blockade augments the cytotoxicity of anti-mitotic drug Taxol™ against breast cancer cells and that this chemosensitizing effect is schedule-dependent. We suggest that the alternate activation of both the pro-apoptotic p38 MAPK-p53 signaling and the cytoprotective MEK1/2 → ERK1/2 cascade, as well as the inactivation of the anti-apoptotic AKT activity may explain, at least in part, the sequence-dependent enhancement of Taxol™-induced cytotoxicity and apoptosis that follows inhibition of FAS activity in breast cancer cells. If chemically stable FAS inhibitors demonstrate systemic anticancer effects of FAS inhibition *in vivo*, these findings may render FAS as a valuable molecular target to enhance the efficacy of taxanes-based chemotherapy in human breast cancer.

Key words: fatty acid synthase; C75; paclitaxel; Taxol™; apoptosis; siRNA; chemotherapy; breast cancer.

A biologically aggressive subset of human breast carcinomas and other malignancies is characterized to exhibit derangement of the *de novo* fatty acid biosynthesis, manifested as overexpression and hyperactivity of the lipogenic enzyme fatty acid synthase (FAS).[1–6] The widespread expression of FAS in human cancer and its association with poorer prognoses in breast,[1–6] ovarian[7,8] and prostate carcinomas[9,10] suggest that high levels of FAS expression and activity provide an advantage for tumor growth and progression.[11] This is in marked contrast to the role of FAS-dependent fatty acid biosynthesis as an anabolic energy storage pathway in liver and adipose tissue. In fact, most human tissues express very low levels of FAS because endogenous fatty acid biosynthesis is downregulated when a normal diet is consumed.[12,13] Interestingly, the differential expression of FAS between cancer and normal tissues has lead to the hypothesis that tumor-associated FAS could be exploited as a useful molecular target for the development of new therapeutic anti-metabolites.[4,14] Specifically, the tumoricidal activity of pharmacological inhibitors of FAS activity has begun to emerge. Thus, it has been demonstrated that cerulenin, [(2R, 3S), 2-3-epoxy-4-oxo-7, 10-*trans*, *trans*-dodecadienamide], a natural product derived from the fungus *Cephalosporium caerulens* that binds irreversibly to the catalytic binding site of the β-ketoacyl carrier protein synthase in the multienzyme FAS complex,[15,16] leads to selective cytotoxicity of cancer cells *in vitro*.[4,14,17–20] *In vivo*, treatment with cerulenin has resulted in significantly increased survival in human cancer xenografts.[21] Unfortunately, the clinical relevance of these results is limited because cerulenin is chemically unstable and may affect processes other than FAS activity. Cerulenin structure harbors a very reactive epoxy group that may interact also with other proteins and may affect processes other than FAS activity, such as protein palmitoylation, cholesterol synthesis or proteolysis.[22–25] Interestingly, a novel inhibitor of FAS has recently become available.[26] The α-methylene-γ-butyrolactone C75 lacks the reactive epoxide present on cerulenin, enhancing chemical stability and specificity. C75 inhibits purified mammalian FAS activity with characteristics of a slow-binding inhibitor and, similarly to cerulenin, it induces cytostatic and cytotoxic effects in cultured tumor cells, and exhibits significant growth inhibitory effects on human breast cancer xenografts.[19,20,26–27]

Although it has become clearer that breast cancer cells are dependent upon active FAS-dependent *de novo* fatty acid synthesis for survival and proliferation, the relationship between breast cancer-associated FAS hyperactivity and the efficacy of chemotherapy has not been studied. We hypothesized that the pharmacological inhibition of FAS activity in human breast cancer cells

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Received 15 May 2004; Accepted after revision 8 October 2004
DOI 10.1002/ijc.20754
Published online 18 January 2005 in Wiley InterScience (www.interscience.wiley.com).

Journal of Cellular Biochemistry 94:857–863 (2005)

VIEWPOINT

Does Endogenous Fatty Acid Metabolism Allow Cancer Cells to Sense Hypoxia and Mediate Hypoxic Vasodilatation? Characterization of a Novel Molecular Connection Between Fatty Acid Synthase (FAS) and Hypoxia-Inducible Factor-1α (HIF-1α)-Related Expression of Vascular Endothelial Growth Factor (VEGF) in Cancer Cells Overexpressing Her-2/*neu* Oncogene

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Abstract Her-2/*neu* (*erb*B-2) oncogene overexpression is associated with increased tumor progression and metastasis. Fatty acid synthase (FAS), the key lipogenic enzyme responsible for the endogenous synthesis of fatty acids, has been shown to be one of the genes regulated by Her-2/*neu* at the level of transcription, translation, and biosynthetic activity. Interestingly, we recently established that both pharmacological inhibition of FAS activity and silencing of FAS gene expression specifically suppress Her-2/*neu* oncoprotein expression and tyrosine-kinase activity in breast and ovarian Her-2/*neu* overexpressors. Unraveling the functional organization of this novel bi-directional molecular connection between Her-2/*neu* and FAS-dependent neoplastic lipogenesis is a major challenge that the field is only beginning to take on. Considering that Her-2/*neu* overexpression correlates with increased expression of the hypoxia inducible factor-1α (HIF-1α), which, in a mitogen-activated protein kinase (MAPK)-dependent manner, plays a key role in the expression of several genes including cytokines such as vascular endothelial growth factor (VEGF), we hypothesized that FAS blockade should result in a concomitant down-regulation of VEGF. Unexpectedly, the specific inhibition of the de novo fatty acid synthesis with the small-molecule inhibitor of FAS activity C75 resulted in a dramatic dose-dependent enhancement (up to 500% increase) of VEGF secretion in Her-2/*neu*-overexpressing SK-Br3, BT-474, and SKOV3 cancer cells. Concurrently, FAS blockade drastically activated MAPK and promoted further a prominent accumulation of HIF-1α in Her-2/*neu* overexpressors. Moreover, U0126-induced inhibition of MAPK activity completely abolished C75-induced up-regulation of HIF-1α expression and VEGF secretion, whereas it did not modulate C75-induced down-regulation of Her-2/*neu* oncogene. Importantly, RNA interference (RNAi)-mediated silencing of the FAS gene recapitulated C75's effects by up-regulating VEGF secretion, MAPK activation and HIF-1α expression. Therefore, it appears that perturbation of cancer-associated endogenous fatty metabolism triggers a "hypoxia-like" (oxygen-independent) condition that actively rescues Her-2/*neu*-dependent MAPK → HIP-1α → VEGF cascade. It is tempting to suggest that an intact FAS-catalyzed endogenous fatty acid metabolism is a necessary metabolic adaptation to support the enhanced ability of Her-2/*neu*-overexpressing cancer cells to survive cellular hypoxia in a HIF-α-dependent manner. J. Cell. Biochem. 94: 857–863, 2005.

Key words: Her-2/*neu*; *erb*B-2; fatty acid synthase; angiogenesis; VEGF; hypoxia; cancer

Her-2/*neu* (*erb*B-2) gene amplification is one of the most consistent alterations found in human malignancies [Akiyama et al., 1986; Slamon et al., 1987]. Although the ultimate biological pathways activated by Her-2/*neu* are not completely characterized, the oncogenic potential of Her-2/*neu* has been consistently

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Received 10 October 2004; Accepted 1 November 2004
DOI 10.1002/jcb.20367

Original article

Annals of Oncology
doi:10.1093/annonc/mdi090

Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* (*erb* B-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin™) in breast cancer cells with Her-2/*neu* oncogene amplification

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Received 7 August 2004; revised 30 October 2004; accepted 4 November 2004

Background: The relationship between the intake of olive oil, the richest dietary source of the monounsaturated fatty acid oleic acid (OA; 18:1n-9), and breast cancer risk and progression has become a controversial issue. Moreover, it has been suggested that the protective effects of olive oil against breast cancer may be due to some other components of the oil rather than to a direct effect of OA.

Methods: Using flow cytometry, western blotting, immunofluorescence microscopy, metabolic status (MTT), soft-agar colony formation, enzymatic *in situ* labeling of apoptosis-induced DNA double-strand breaks (TUNEL assay analyses), and caspase-3-dependent poly-ADP ribose polymerase (PARP) cleavage assays, we characterized the effects of exogenous supplementation with OA on the expression of Her-2/*neu* oncogene, which plays an active role in breast cancer etiology and progression. In addition, we investigated the effects of OA on the efficacy of trastuzumab (Herceptin™), a humanized monoclonal antibody binding with high affinity to the ectodomain of the Her-2/*neu*-coded $p185^{Her\text{-}2/neu}$ oncoprotein. To study these issues we used BT-474 and SKBr-3 breast cancer cells, which naturally exhibit amplification of the Her-2/*neu* oncogene.

Results: Flow cytometric analyses demonstrated a dramatic (up to 46%) reduction of cell surface-associated $p185^{Her\text{-}2/neu}$ following treatment of the Her-2/*neu*-overexpressors BT-474 and SK-Br3 with OA. Indeed, this effect was comparable to that found following exposure to optimal concentrations of trastuzumab (up to 48% reduction with 20 μg/ml trastuzumab). Remarkably, the concurrent exposure to OA and suboptimal concentrations of trastuzumab (5 μg/ml) synergistically down-regulated Her-2/*neu* expression, as determined by flow cytometry (up to 70% reduction), immunoblotting, and immunofluorescence microscopy studies. The nature of the cytotoxic interaction between OA and trastuzumab revealed a strong synergism, as assessed by MTT-based cell viability and anchorage-independent soft-agar colony formation assays. Moreover, OA co-exposure synergistically enhanced trastuzumab efficacy towards Her-2/*neu* overexpressors by promoting DNA fragmentation associated with apoptotic cell death, as confirmed by TUNEL and caspase-3-dependent PARP cleavage. In addition, treatment with OA and trastuzumab dramatically increased both the expression and the nuclear accumulation of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor playing a key role in the onset and progression of Her-2/*neu*-related breast cancer. Finally, OA co-exposure significantly enhanced the ability of trastuzumab to inhibit signaling pathways downstream of Her-2/*neu*, including phosphoproteins such as AKT and MAPK.

Conclusions: These findings demonstrate that OA, the main monounsaturated fatty acid of olive oil, suppresses Her-2/*neu* overexpression, which, in turn, interacts synergistically with anti-Her-2/*neu* immunotherapy by promoting apoptotic cell death of breast cancer cells with Her-2/*neu* oncogene

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Original article

Annals of Oncology 16: 1253–1267, 2005
doi:10.1093/annonc/mdi239
Published online 3 May 2005

Antitumoral actions of the anti-obesity drug orlistat (Xenical™) in breast cancer cells: blockade of cell cycle progression, promotion of apoptotic cell death and *PEA3*-mediated transcriptional repression of Her2/*neu* (*erb* B-2) oncogene

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Received 26 December 2004; accepted 6 March 2005

Background: Orlistat (Xenical™), a US Food and Drug Administration (FDA)-approved drug for bodyweight loss, has recently been demonstrated to exhibit antitumor properties towards prostate cancer cells by virtue of its ability to block the lipogenic activity of fatty acid synthase (FAS). FAS (oncogenic antigen-519) is up-regulated in about 50% of breast cancers, is an indicator of poor prognosis, and has recently been functionally associated with the Her2/*neu* (*erb* B-2) oncogene.

Materials and methods: We assessed the antitumoral effects of orlistat against the human breast cancer cell line SK-Br3, an *in vitro* paradigm of FAS and Her2/*neu* overexpression in breast cancer.

Results: Cell cycle analyses revealed that micromolar concentrations of orlistat induced, in a time- and dose-dependent manner, significant changes in the distribution of cell populations including a complete loss of G_2-M phase, S-phase accumulation and a concomitant increase in the emerging sub-G_1 (apoptotic) cells. Poly (ADP-ribose) polymerase (PARP) cleavage, an early event required for cells committed to apoptosis, was more predominant in orlistat-treated G_1 phase cells. When we characterized signaling molecules participating in the cellular events following orlistat-induced inhibition of FAS activity and preceded inhibition of breast cancer cell proliferation, a dramatic down-regulation of Her2/*neu*-coded $p185^{Her2/neu}$ oncoprotein was found in orlistat-treated SK-Br3 cells (>90% reduction). Interestingly, a significant accumulation of the DNA-binding protein PEA3, a member of the *Ets* transcription factor family that specifically targets a PEA3-binding motif present on the Her2/*neu* gene promoter and down-regulates its activity, was observed in orlistat-treated SK-Br3 cells. When a Luciferase reporter gene driven by the Her2/*neu* promoter was transiently transfected in SK-Br3 cells, orlistat exposure was found to dramatically repress the promoter activity of Her2/*neu* gene, whereas a Her2/*neu* promoter bearing a mutated binding DNA sequence was not subject to negative regulation by orlistat, thus demonstrating that the intact PEA3 binding site on the Her2/*neu* promoter is required for the orlistat-induced transcriptional repression of Her2/*neu* overexpression. RNA interference (RNAi)-mediated silencing of FAS gene expression similarly repressed Her2/*neu* gene expression in a PEA3-dependent manner, thus ruling out a role for non-FAS orlistat-mediated effects. When the combination of orlistat and the anti-Her2/*neu* antibody trastuzumab (Herceptin™) in either concurrent (orlistat + trastuzumab) or sequential (orlistat → trastuzumab; trastuzumab → orlistat) schedules was tested for synergism, addition or antagonism using the combination index (CI) method of Chou–Talalay, co-exposure of orlistat and trastuzumab demonstrated strong synergistic effects (CI_{10-90} = 0.110–0.847), whereas sequential exposure to orlistat followed by trastuzumab (CI_{10-90} = 0.380–1.210) and trastuzumab followed by orlistat (CI_{10-90} = 0.605–1.278) mainly showed additive or antagonistic interactions. Indeed, orlistat-induced FAS inhibition synergistically promoted apoptotic cell death when concurrently combined with trastuzumab as determined by an ELISA for histone-associated DNA fragments. Importantly, the degree of FAS expression in a panel of human breast cancer cell lines was predictive of sensitivity to orlistat-induced anti-proliferative effects as determined by a MTT-based characterization of

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